TITLE

What uniparental genetic markers tell us about the prehistoric human colonization of the Americas

AUTHOR

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Running title

Uniparental markers and the American colonization

ABSTRACT

From the perspective of uniparental markers, the view of the human prehistoric settlement of America is that it resulted from a single main migration after the Last Glacial Maximum, following a period of prolonged stasis in Beringia. These pioneers rapidly expanded across a continent previously uninhabited by humans, forming isolated population groups that became genetically distinct. Ancient DNA studies and whole genome analyses have confirmed this view, revealing a remarkable complexity in the ancestries of the involved populations.

In this work, I have reanalyzed all publicly available mitochondrial DNA and Y-chromosome haplogroups in the American population using simple phylogenetic and phylogeographic methodologies.
The results provide a genetic perspective opposed to the currently established model. The arrival of the American settlers occurred more than 30,000 years ago, preceding the LGM. As genomic studies have uncovered, at least two Asian populations contributed to the ancestry of the immigrant population. Low population density and climatic deterioration led to a long period of demographic eclipse, during which small bands of hunter-gatherers made long journeys in search of favorable niches. After the LGM, the climate improved, and demographic expansions occurred in multiple independent centers. The founding and expansion ages of the uniparental haplogroups indicate that these centers were in South America, particularly the Colombian isthmus, the Andean region, the Southern Cone, and the Amazon. Subsequent dispersals occurred in North America, one involving mitochondrial haplogroups A2, C1, and D1, but not B2, and another, of lesser magnitude, represented by the expansion of haplogroups C4c and X2a.
This work offers a previously unexplored model for the colonization of the Americas.

INTRODUCTION

The interpretation of the prehistoric peopling of the Americas has been a multidisciplinary enterprise (Becerra-Valdivia and Higham 2020; Fiedel 2000). From a molecular genetics perspective, the first foundations of these interpretations are based on the information provided by the uniparentally inherited mitochondrial DNA (mtDNA) and the non-recombining portion of the Y-chromosome (NRY) on extant populations (Schurr 2004; Karafet et al., 1999). As ancient DNA (aDNA) samples are a mixture of endogenous aDNA and contaminating DNA, results on human samples were first questioned. Fortunately, new sequencing technologies have overcome this problem (Palencia-Madrid and De Pancorbo 2015), and current aDNA-based analyses have added an invaluable timing perspective to these interpretations (Bisso-Machado and Fagundes 2021). Later, technological improvements have made it possible to sequence entire genomes in present-day and fossil individuals. Analyses of these genomes have revealed extinct ancestries and genetically well-differentiated ancestries from the past that, admixed in different proportions, persist in current Native American populations along the American continent. This new information has improved previous hypotheses and formulated new ones (Willerslev and Meltzer 2021).

Thus, some conclusions have achieved general acceptance: 1) The Asiatic origin of the Native American ancestors; 2) The first migration, in one or several waves, occurred as a rapid movement towards the south of the double continent; 3) The initial low population density led to strong genetic differentiation geographically structured; 4) Subsequent migrations reshaped the genetic landscape of North and South America. Necessary for the first was the entrance from Beringia of several migratory waves that originated the settlement of Paleo- and Neo-Eskimos; 5) Time comparisons of population genetic profiles since post-Neolithic to present-day have uncovered an essential genetic continuity (although see (Llamas et al. 2016)).
However, other propositions are still under debate, such as: 1) the precise Asian origin of the Native American ancestors; 2) the routes followed by these pioneers as they expanded throughout the Americas; and, perhaps, the most contentious question, 3) when these first migrations occurred? Although the most recent studies based on complete genomes favor a post-Glacial dispersal (Moreno-Mayar et al. 2018), with or without a prolonged standstill in Beringia (Faught 2017), the first works with mitochondrial DNA favored an early entry, around 30 kya before the Last Glacial Maximum (LGM), avoiding the problem of having to cross the ice sheets of North America (Bonatto and Salzano 1997; Torroni et al. 1994). These age discrepancies are due to the molecular evolutionary rates used in each case. Unfortunately, the rate of molecular evolution is not constant; it depends on several parameters, one of which is the adequate population size (Ne), which has changed dramatically from the Paleolithic to present-day (Cabrera 2020). Therefore, like radiocarbon dating, molecular rates must be calibrated to obtain more accurate ages. One way to do this is by using ancient genomes and/or mitogenomes, but in the case of the American colonization, all the extracted aDNA postdates the LGM (Bisso-Machado and Fagundes 2021). The option is to use evolutionary rates calibrated with Paleolithic fossils to date Paleolithic events (Cabrera 2024).
Despite their utility, whole-genome studies barely mention uniparental markers (Gusareva et al. 2025). But see (Moreno-Mayar et al. 2018; Krettek et al. 2025). The underlying reason for this abandonment is that these markers only offer monogenic insights, and only the set of all genes provides congruent results. In my opinion, this assumption is flawed. The leading cause of genetic differentiation in estimating phylogenies and genetic distances between populations or species is isolation. Isolation affects whole genomes and does not discriminate by sex. Populations differentiate genetically, due to the loss of previous polymorphisms, by fixation or extinction, and the accumulation of new polymorphisms by mutation in a time-dependent way. Thus, any DNA fragment, including those coding for uniparental markers, should be valid to

realize genetic comparisons whenever they have accumulated enough genetic differentiation. The generalized use of DNA barcoding in molecular taxonomy (Hubert and Hanner 2015) validates my previous assertion. Isolation is broken by migration/introgression, affecting uniparental markers and autosomes differently. Whether uniparental markers persist only in their original populations or both endure in the original and recipient populations, their coalescence node—the distance to their most recent common ancestor—will remain unchanged. However, if the introgressed fragments are not thoroughly masked for mixed autosomes, they will influence the genetic distances obtained between the populations/species involved. A paradigmatic example is the controversial phylogenetic relationships obtained between modern humans and their closest relatives, Neanderthals and Denisovans. While mtDNA and NRY sequences showed modern humans and Neanderthals as sister lineages (Posth et al. 2017; Petr et al. 2020), autosomes joined Denisovan and Neanderthal as the closest pair, leaving modern humans as an out-group (Posth et al. 2017). Contrary to the widespread acceptance that the proper relationship between hominids is that obtained with the autosomes, I have suggested that the valid phylogeny is deduced from the uniparental markers. In contrast, the one obtained from the autosomes results from later introgressions between the three groups of hominins (Cabrera 2021b).
Taking into account the above-mentioned particularities of the uniparental markers, in this work, I perform a thorough reanalysis of all published mitogenomes and the most recently published Y-chromosome results, using phylogenetic and phylogeographic tools, to reconsider and shed new light on the hypotheses formulated to explain the prehistoric colonization of the New World by Asian peoples. The main conclusions obtained are: 1) that the first migration involved at least two Asian populations with most probable central and eastern Asian origins; 2) that this wave(s) occurred before 30 Kya; 3) that those pioneer colonizers did not stop in Beringia but kept trekking across the Americas leaving few genetic traces in past and present-day native American populations; 4) That after the LGM, small and relatively isolated resilient groups radiated from different areas to occupy new exploitable territories both in North and South America; 5) that these secondary expansive waves occurred first in southern- an meso-America than in northern North America; and 6) that gene flow existed among geographically adjacent groups.

RESULTS

All the phylogenetic and phylogeographic mtDNA results described below have been obtained from the trees presented in the supplementary figures

(Supplemental_Fig_S1.xls (HgA2), Fig_S2.xls (HGB2), Fig_S3.xls (HgC1), Fig_S4.xls (HgD), and Fig_S5.xls (HgX2), and all the NRY results described below have been obtained from the trees already published by other authors (Wei et al. 2018; Grugni et al. 2019; Pinotti et al. 2019; Villaescusa et al. 2021; Colombo et al. 2022; Paz Sepúlveda et al. 2022).

The most probable origin of the American Asian ancestors

The main Native American mtDNA haplogroup founders can be divided into two groups: 1) those belonging to macro-Haplogroup M (C1, C4c, D1, D2a1, D4e1b, and D4h3a), and 2) those that belong to macro-Haplogroup N (A2, B2, and X2a). The same grouping occurs when we look at the number of mutations accumulated in their basal stems that link them to the common nodes with Asian lineages. While C1, C4c, D1, D2a1 and D4h3a have two mutations, and D4e1b only one; A2 and B2 have five mutations and X2a four mutations (Table 1).

Table 1. Mutations on American basal branches to their common nodes with Asia

American haplogroup	**Mutations**	**Coalescent Age (Kya)**	**Asian haplogroup**	**Asian region**			
A2	5	20,2	A-a1	NW China (Xinjiang)			
A10a	1	4,04	A10	Western Siberia			
B2	5	20,2	B4b1a	Altai			

C1	2	8,08	C4	Eastern Asia			
C4c	2	8,08	C4a'b'c	Eastern Asia			
D1	2	8,08	D4	Eastern Asia			
D4e1b	1	4,04	D4e1	Eastern Asia			
D2a1	2	8,08	D2a'b	Northeastern Siberia			
D4h3a	2	8,08	D4h3	NW China (Xinjiang)			
X2a	4	16,16	X2a'j	Tajikistan			

An unpaired t-test shows that the difference between groups is highly significant (t = 7.2164; df = 5; two-tailed P value = 0.0008). Furthermore, it is well known that macro-Haplogroup M is dominant in southern and eastern Asia (Marrero et al. 2016), while macro-Haplogroup N is more frequent in western Eurasia (Fregel et al. 2015). Consequently, the same occurs with the Asian ancestral sequences/haplogroups that show the shortest distances with the Native American haplogroups. It has already been demonstrated that the closest relatives for the macro-Haplogroup M American lineages are found in eastern Asia/southeastern Siberia (Starikovskaya et al. 1998; Derenko et al. 2010). Some further commentaries are pertinent for these lineages: Hg C1 comprises four common and well spread sub lineages, three of them (C1b, C1c, C1d) practicably exclusive of the Americas and one (C1a) with a wide Asian range, and several rare clades/isolates, the most ancient of which is represented by two identical sequences from Brazil (Simão et al. 2021) and

Paraguay (Avila et al. 2019) that carries twelve mutations in its basal stem (Supplementary_Fig_S3.xls). Curiously, the other isolates have been detected in northwestern Eurasia instead of East Asia: C1e in Iceland (Ebenesersdóttir et al. 2011), western Russia (Järve et al. 2019), and western China (Wang et al. 2021); C1f in Tajikistan (Peng et al. 2018) and C1g in Mesolithic remains from western Russia (Der Sarkissian et al. 2014). As the oldest clades are found in the New World, the most parsimonious conclusion is that all the C1 branches in Eurasia resulted from retro migrations from America. The oldest Native American samples harboring C1b lineages are USR1 from Alaska (Tackney et al. 2015) and I11974 from Chile (Posth et al. 2018), both around 12,000 years old; for C1c it is MW291667 from Argentina (Roca-Rada et al. 2021), approximately 8,500 years old; and for C1d it is Lapa01, 9,600 years old, from Brazil (Posth et al. 2018). Haplogroup D1 has the oldest samples in Yucatan, Mexico, represented by a Late Pleistocene skeleton (HN5/48) around 12 ky old (Chatters et al. 2014), in Spirit Cave, Nevada, around 11 kya (Moreno-Mayar et al. 2018), an approximately 10 ky old LLP.S2.E1 sample from Argentina (Roca-Rada et al. 2021), and the Sumidouro4, 9,645 years old, sample from Brazil (Moreno-Mayar et al. 2018). Until recently, the American haplogroup D4h3a was separated from its Asian root by six mutations, but recent aDNA studies (Mao et al. 2021) have rescued a Chinese sequence that shares four of the six basal substitutions with the American clade (Supplementary_Fig_S4.xls). The remains, from which this sequence was extracted, were unearthed at the Amur region, in northeastern China, and are around 14,000 years old. The oldest samples harboring D4h3a sequences in the New World are the 12,600-year-old Anzick1 from Montana (Rasmussen et al. 2014), the 10,300-year-old Shuká Káa from Alaska (Lindo et al. 2017), and the 10,400-year-old specimens from Lagoa Santa in Brazil (Moreno-Mayar et al. 2018).

About the macro-Haplogroup N, for the New World haplogroup A2, the closest Asian sequences are from haplogroup A-a1 (BZL_M16A, BZL_M16B) and belong to historic samples from Xinjiang, western China (Wang et al. 2021). For haplogroup B2, it is AY519494, included in the Asian haplogroup B4b (Supplementary_Fig_S2.xls), with the Altai region as its geographic origin (Starikovskaya et al. 2005). Finally, for the American haplogroup X2a, the closest Asian relatives are into haplogroup X2j, that groups samples from Egypt and Europe (Supplementary_Fig_S5.xls) but, for geographic proximity, I consider as the closest Asian sequence a partial one (sample 40) from Tajikistan (Ovchinnikov et al. 2014), as it carries 16179 and 16357 substitutions that are terminal mutations in the Egyptian X2j (Kujanová et al. 2009) mitogenomes (Supplementary_Fig_S5.xls). For haplogroups A2 and B2 the most ancient sequences, around 14,000 years old, were extracted from human

coprolites found in Paisley Caves, Oregon (Gilbert et al. 2008), next oldest for A2 is Lapa15 (around 8,700 years old) from Lapa do Santo, Brazil (Posth et al. 2018), and for B2 is USR2 (approximately 12,000 years old) from Upward Sun River site in Alaska (Tackney et al. 2015). The most ancient Native American sample with an X2a haplotype is the Kennewick Man from Washington, which is around 9,000 years old (Rasmussen et al. 2015).

A way to reconcile the close arrivals of the Asian ancestors carrying the Native American M and N lineages with their significant differences in the number of mutations accumulated on their basal stems is to assume that the route followed across Asia of the N carriers was longer and slower than that of the M carriers.

Finally, if we compare the number of lineages carried by the M and N groups to their respective population sizes upon arrival in America, we could estimate that the contribution of the eastern Asian contingent was 62.5% and that of western Eurasia 37.5%.

The NRY does not show American colonization patterns as clear as those of mtDNA, but using its phylogeny and phylogeography in the Americas and Asia I have considered the ancestors of the American clades C-MPB373, that has its closest haplotypes in the eastern Asian outgroup clade C-L1373 (Pinotti et al. 2019; Sun et al. 2021), QF746 that includes the Q-YP1475 subclade represented by the Saqqaq ancient sample (Rasmussen et al. 2010) and has as sister subclade Q-B143 represented by northeastern Siberian Koryaks (Karmin et al. 2015), and Q-FT9713, that has its closest sequences in Mongolia and northeastern China (https://www.yfull.com/tree/Q) as of eastern Asian origin, while C-P39/Z30536, having as outgroup the Central Asian subclade C-F1756 (Wei et al. 2018), Q-Z780, that includes the Native American ancient sample Anzick1 (Rasmussen et al. 2014) and has Q-L330 of Central Asia ascription (Dulik et al. 2012; Flegontov et al. 2019) as sister clade, Q-M3, that has as out group Q-L804 with a northern European geographic range (Hallast et al. 2015), and the rare Q-Y1150 and Q-M378 (Grugni et al. 2019) as of western Eurasian origin. Unlike mtDNA, most American male lineages appeared to have their ancestors in western Eurasia. It is interesting to note that the C-MPB373 South American lineage is phylogenetically an outgroup of C-F1699 that comprises numerous western Eurasian lineages, including also the Canadian C-P39/Z30536 branch (Pinotti et al. 2019). Also of interest is that the most abundant American clade, Q-M3 (Colombo et al. 2022), has as sister branch Q-L804, which is specific to northern Europe (Grugni et al. 2019). The oldest sample belonging to the eastern group of lineages is Lapa01, from Brazil, which is approximately 8,840 years old (Posth et al. 2018). For the western group, Anzick1 from Montana, with an approximate age of 12,600 years, represents

the oldest North American sample (Grugni et al. 2019). In contrast, the oldest sample (I11974) in South America was found in Los Rieles, Chile, and is approximately 10,500 years old (Posth et al. 2018).

The global ages of the first arrival and expansions of the Native American parental ancestors

A global average of the different mean mutational data, calculated for each American mitochondrial haplogroup using various approaches (Table 2), give mean mutational values of 7.11 (95% CI: 6.34 – 7.88) substitutions at the arrival in America of the founding lineages, and a mean of 4.42 (95%: 3.98 – 4.85) substitutions for their initial expansions into the American continent, which, in turn, produce mean ages of 28.72 (95%: 25.61 – 31.84) kya and of 17.86 (95% CI: 16.08 – 19.59) kya for the founding and expansion events respectively.

Table 2. Mutational means and coalescent mean ages for the main American mtDNA haplogroups

HG	**Phylogeny**	**Mean (Age Kya)**	**Type**	**N**	**95% CI: Mutation/Age(Kya)**			
A2	Founding	7.55 (30,50)	Normal	1119 (Indiv)	7.37-7.73 / 29,77-31,23			
	Expansion	4.63 (18,70)	"	1083 (Indiv)	4.46-4.79 / 18,02-19,35			

	Founding	7.25 (29,29)	Weighted	118 (Clades)	6.62-7.88 / 26,74-31,83			
	Expansion	4.35 (17,57)	"	118 (Clades)	4.04-4.66 / 16,32-18,83			
	Coalescent	9.13 (36,88)	Max (19 nt)	16 (Branches)	8.46-9.79 / 34,18-39,55			
B2	Founding	6.68 (26,99)	Normal	1081 (Indiv)	6.51-6.85 / 25,30-27,68			
	Expansion	4.68 (18.90)	"	1070 (Indiv)	4.51-4.85 / 18,22-19,00			
	Founding	6.62 (26,75)	Weighted	80 (Clades)	6.21-7.03 / 25,09-28,40			
	Expansion	4.69 (18,95)	"	80 (Clades)	4.28-5.11 / 17,29-20,64			

	Coalescent	6.42 (25,93)	Max (16 nt)	14 (Branches)	5.12-7.72 / 20,68-31,17			
C1	Founding	6.50 (26,26)	Normal	887 (Indiv)	6.33-6.67 / 25.57-26,95			
	Expansion	4.36 (17,61)	"	873 (indiv)	4.17-4.55 / 16,84-18,38			
	Founding	6.33 (25,57)	Weighted	138 Clades)	6.05-6.61 / 24,44-26,71			
	Expansion	4.01 (16,20)	"	138 (Clades)	3.71-4.31 / 14,99-17,41			
	Coalescent	6.22 (25,13)	Max (16 nt)	42(Branches)	5.54-6.90 /22,38-27,88			
C4c	Founding	6.32 (25,53)	Normal	34 (Indiv)	5.74-6.91 /23,19-27,92			

	Expansion	4.32 (17,45)	"	34 (Indiv)	3.74-4.91 / 15,11-19,84			
	Founding	3.48 (14,06)	Weighted	8 (Clades)	2.85-4.11 /11,51-16,60			
	Expansion	1.30 (5,25)	"	8 (Clades)	0.67-1.93 /2,71-7,80			
	Coalescent	4.71 (19,03)	Max (9 nt)	3 (Branches)	2.47-6.96 /9,98-28,12			
D1	Founding	7.00 (28,28)	Normal	496 (Indiv)	6.73-7.27 / 27,19-29,37			
	Expansion	4.97 (20,08)	"	463 (Indiv)	4.68-5.27 / 18,91-21,29			
	Founding	7.02 (28,36)	Weighted	58 (Clades)	6.53-7.51/26,38-30,34			

	Expansion	4.97 (20,08)	"	58 (Clades)	4.48-5.46 / 18,10-22,06			
	Coalescent	7.14 (28,86)	Max (16 nt)	28(Branches)	6.67-7.62 / 26,93-30,79			
D4h3a	Founding	6.20 (25,05)	Normal	80 (Indiv)	5.57-6.83 / 22,50-27,59			
	Expansion	4.24 (17,13)	"	64 (Indiv)	3.63-4.85 /14,67-19,59			
	Founding	6.27 (25,33)	weighted	13 (Clades)	5.73-6.81 / 23,15-27,51			
	Expansion	4.24 (17,13)	"	13 (Clades)	3.80-4.68 / 15,35-18,91			
	Coalescent	7.77 (31.39)	Max (12 nt)	12(Branches)	6.15-9.39 / 24,85-37,93			
X2a	Foundi	7.83	Normal	41 (Indiv)	7.11-8.55 / 28,72-			

	ng	(31,63)			34,54			
	Expansion	3.83 (15,47)	"	40 (indiv)	3.11-4.55 / 12,56-18,38			
	Founding	6.83 (27,59)	Weighted	2 (Clades)	5.37-8.29 /21,69-33,49			
	Expansion	2.83 (11,43)	"	2 (Clades)	1.37-4.29 / 5,53-17,33			
	Coalescent	11.00 (44,44)	Max (15 nt)	2(Branches)	-39.82-61.82/-160,87-249,75			

The founding ages for the mtDNA Native American haplogroups, exposed above, coincide with the approximately 30 kya proposed in the first mtDNA studies (Bonatto and Salzano 1997; Torroni et al. 1994; Starikovskaya et al. 1998), and with the calibrated ages calculated by (Rieux et al. 2014), based on ancient mitogenomes. However, recent extant and ancient mitogenome studies (Bisso-Machado and Fagundes 2021; Llamas et al. 2016) and whole genome-based studies (Moreno-Mayar et al. 2018; Willerslev and Meltzer 2021) strongly support post-LGM ages for the settlement of the Americas,

although this is in part inspired by the most conservative archaeological ages proposed for the American settlement.

The age for the first population expansions calculated here, 17.86 kya, after the rigors of the LGM had lessened on the continent, does not differ much from those proposed by most authors. The difference is that this expansion coincides with most authors' arrival in America proper. In contrast, here, the arrival in America occurred much earlier, around 30 kya, before the glacial period had its worst effects.

Concerning the NRY, it deserves mention that, assuming a first colonization of the Americas about 15 kya (Poznik et al. 2013), estimated a substitution rate for the Y-chromosome of 8.2 x 10-10 (95% CI: 7.2 x 10-10 – 9.2 x 10-10) per site, year. However, doubling the time to 30 kya, as proposed here based on mtDNA, the substitution rate would be 4.1 x 10-10 per site, year which is very similar to that obtained by (Francalacci et al. 2013) of 5.3 x 10-10 (95% CI: 4.2 X10-10- 7.0 x 10-10) per site, year and both estimates falling within the limits of the mean 4.7 x 10-10 (95% CI: 2.1 X 10-10 – 9.5 X 10-10) per site, year proposed by myself based on past population sizes obtained under phylogenetic considerations (Cabrera 2024). As before, these results contrast with the majority of the NRY younger coalescence ages estimated by other authors (The Genographic Consortium et al. 2016; Pinotti et al. 2019; Colombo et al. 2022; Paz Sepúlveda et al. 2022).

As YFull Tree v13.03.00 (YTree) has the most actualized NRY phylogeny and the most extensive phylogeography, it is increasingly used as a data source in scientific articles, as in the present work. The YFull substitution rate (Adamov et al. 2015) is identical to the one calculated by (Poznik et al. 2013) under the premise that the human American settlement occurred around 15 kya, because of this, I have halved their substitution rate values when I used them. Under these requirements, NRY haplogroup C clades would arrive in the Americas in separate waves, C-MPB373 first at about 32.2 kya (95% CI: 28.8 – 35.6 kya), and C-P39/Z30536 second at about 26.2 kya (95% CI: 22.8 – 29.8 kya). The three main NRY haplogroup Q clades have very similar ages around 30 kya: Q-F746, 31.8 kya (95% CI: 28.4 – 35.2); Q-Z780, 31.0 kya (95% CI: 29.0 – 33.0); Q-M3, 29.8 (27.0 – 32.8). The age of arrival to the Americas of the rare clades Q-M1150 (30.0 kya (95% CI:26.6 – 33.4 kya) and Q-M378, 21.4 kya (95% CI: 17.6 – 25.4 kya), that include European and Asian lineages, is only tentative (https://www.yfull.com/tree/Q).

Regional heterogeneity for founding and expansion events

Initially, I compared the founding and expansion ages of native haplogroups between North and South American subcontinents (Table 3). Except for haplogroup C1, the results show significantly older ages in South America for both founding and expansion events.

Table 3. Differences in Founding(F) and expansion(E) ages between the North America and South America subcontinents

Haplogroup	t-value	df	p-value	oldest region
A2 - F	3.0397	1119	0.0024	South America
A2 -E	4.8326	1105	<0.0001	South America
B2 - F	1.0604	1099	0.2892	ns
B2 - E	3.1395	1083	0.0017	South America
C1 - F	1.4777	499	0.1401	ns
C1- E	0.4722	482	0.6370	ns

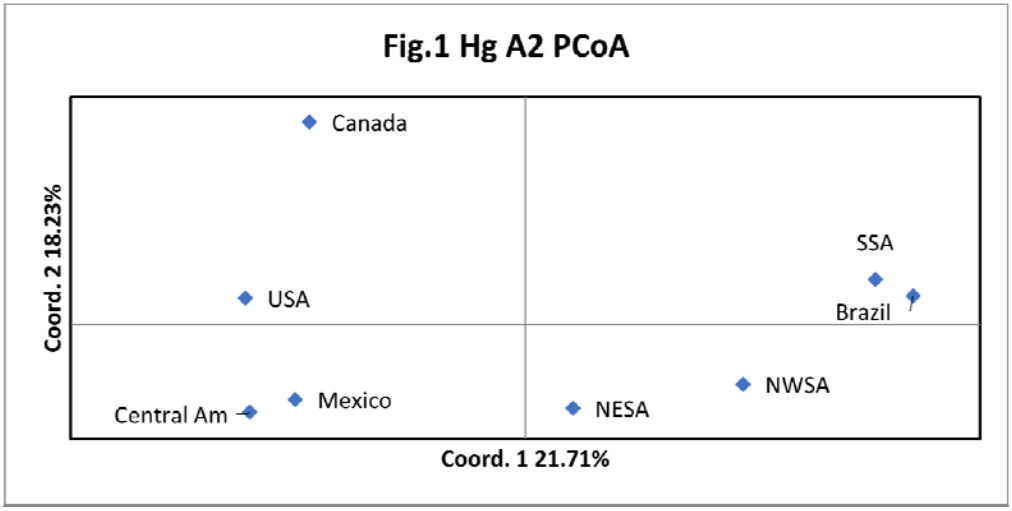
D1 - F	5.7700	498	<0.0001	South America							
D1 - E	2.2473	466	0.0251	South America							
D4h3a - F	3.0245	69	0.0035	South America							
D4h3a - E	1.0115	59	0.3159	ns							

More detailed comparison between geographic regions, within and among subcontinents, using mean molecular age comparisons, pairwise genetic distances and Pearson correlation tests are described in supplementary tables (Supplemental Table S1.1 to S1.7 for A2; Supplemental Table S2.1 to S2.7 for B2; Supplemental Table S3.14, .7, .10, .13, .16 for C1b; Supplemental Table S3.2, .5, .8, .11, .14, .17 for C1c; Supplemental Table S.3.3, .6, .9, .12, .15, .18 for C1d; Supplemental Table S3.19 for C4c; Supplemental Table S4.1 to S4.6 for D1; Supplemental Table S4.7 to S4.9 for D4h3, and Supplemental Table S5.1 to S5.3 for X2a).

Haplogroup A2

Globally, A2 is the most abundant haplogroup, representing approximately 44% of the Native American population, but it has an uneven geographic distribution. The oldest founding ages for A2 (Supplemental_Table_S1.5) are in Brazil and the Colombia-Isthmus area (≈ 34 kya), expanding first in Colombia around 23 kya. From the Colombian Isthmus region, significant latitudinal clines exist to Siberia (Slope -13.37; R^2 0.8396; P-value 0.0102), and to Tierra del Fuego (Slope 23.49; R^2 0.8091; P-value 0.0377). In addition, notice that in the most ancient samples of southern South America, mtDNA haplogroup A and B lineages were totally absent or very rare (Lalueza 1997; Moraga et al. 2000; Nakatsuka et al. 2020; Roca-Rada et al. 2021; Postillone et al. 2020) while they were detected in the most ancient samples recovered in northwestern North America more than 14 kya (Gilbert et al. 2008), and also were prominent in ancient samples from the Isthmus-Colombian area

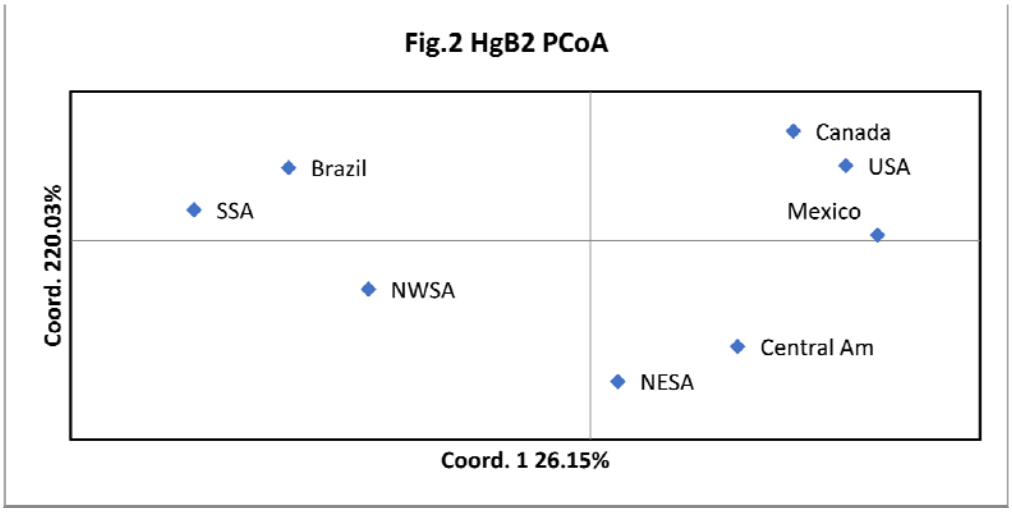
(Capodiferro et al. 2021). Another geographic peculiarity of haplogroup A2 is its significant abundance in the eastern regions compared to the western ones (Kemp et al. 2007; Raff et al. 2011; Rothhammer et al. 2017; Simão et al. 2021). Notably, the oldest A2 haplotypes are found in North America and Brazil (Table S1.4). Furthermore, a secondary southward migration of some North American sub-lineages is also detectable. One of these lineages is A2f, which is significantly older in North America (≈35,000 ya) than in Mexico (≈23,000 ya) (t=3.6172; df 22; p-value 0.0015).
For haplogroup A2, the shortest molecular genetic distances between regions (Table S1.3) were found for the pairs CSAm–SSAm (0.657), NWSAm–NESAm (0.682), and NCAm–NNAm (0.681). When these distances are shown in a PCoA (Fig. 1), the first principal coordinate places NESAm in a central position, with the remaining regions of North- and South-America positioned to its left and right, respectively. The second principal coordinate separates the areas of each subcontinent as latitudinal clines, reflecting the Pearson correlation results.



Haplogroup B2

Haplogroup B2 is the second-largest Native American haplogroup, comprising approximately 24.0% of the global distribution. The oldest founding (approximately 30,000 years ago) and expansion (approximately 21,000 years ago) regional values for this haplogroup are found in South and Central America. As one moves northward from these southern regions, the values decrease in a clinal trend, following a northwest-southeast orientation to reach Central and North America. A significant negative correlation exists between

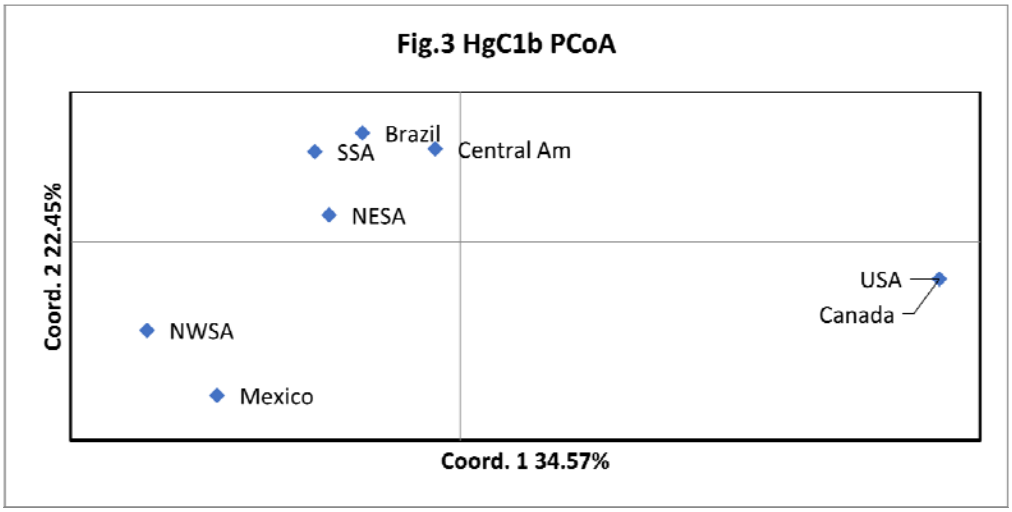
the molecular ages of haplogroup B2 and latitude (Slope: -13.57; R^2: 0.5455; P-value: 0.0061).
Unlike haplogroup A2, haplogroup B2 is either absent or rare in northern North America, which mirrors its low frequency in northeastern Siberia (Starikovskaya et al. 1998). Moreover, in contrast to haplogroups A2 and D1, haplogroup B2 is more prevalent in western continental regions than in eastern ones (Bisso-Machado et al. 2012; Brandini et al. 2018). The oldest B2 haplotypes have been identified in the Andean region.
The shortest genetic molecular distances were observed between the South South American (SSAm) and Central South American (CSAm) groups (0.492), as well as between the SSAm and Northwest South American (NWSAm) pairs (0.500). A Principal Coordinates Analysis (PCoA) represented these distances graphically, showing NWSAm in a central position, with SSAm and CSAm on the left and Northeastern South American (NESAm) and all northern American regions on the right. This arrangement follows a latitudinal cline, as confirmed by the Pearson correlation (Fig. 2). (Supplementary_Table_ S2.3). A PCoA bidimensional graphic representation of these distances shows NWSAm having a central position, with SSAm and CSAm at its left and NESAm and all northern American regions at its right, following a latitudinal cline as observed with the Pearson correlation (Fig. 2).



Haplogroup C1

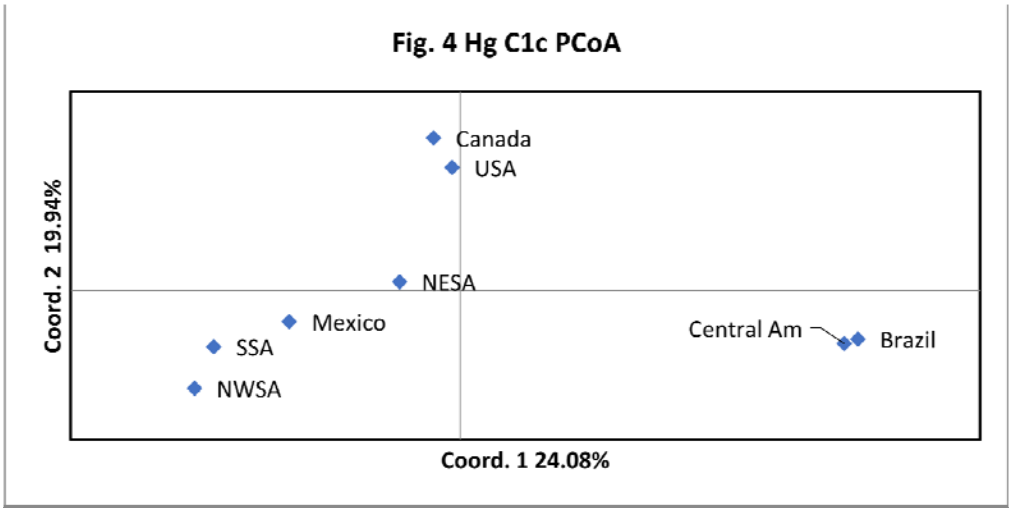
Haplogroup C1 was initially considered a homogenous clade; however, its three main native branches reflect distinct phylogeographic movements that warrant particular attention.
For haplogroup C1b, the oldest founding and expansion regional ages (see Supplementary Table S3.13) are found in Northeast South America

(approximately 29,000 to 17,000 years ago), with younger ages extending down to Tierra del Fuego and up to Canada. The data shows a significant trend (Slope -12.20; R^2 0.8876; P-value 0.0166). These phylogeographic characteristics are somewhat similar to those of haplogroup A2. However, the longitudinal differentiation seen in the A2 clade is not present in C1b. The oldest haplotypes for C1b have been identified in northern South America, particularly in Ecuador and Colombia, and extend into Mexico (see Supplementary Table S3.10). Additionally, the shortest genetic distance between regions (refer to Supplementary Table S3.7) is observed between Northwest South America (NWSAm) and Northeast South America (NESAm) with a distance of 0.522, followed by NWSAm and Southern South America (SSAm) at 0.605, and SSAm to Central South America (CSAm) at 0.654.
In this context, the PCoA (Principal Coordinates Analysis) graphic representation emphasizes the close relationship between NWSAm–Mexico and the USA–Canada regions, positioning NESAm as the central point from which movements occurred both southward across Brazil and northward into Central America (see Fig. 3).



For Hg C1c, the earliest regional ages associated with founding and expansion (Supplementary_Table_ S3.14) are centered in NWSAm (≈ 34 kya / 26 kya). From this center, populations with younger ages follow southern and northern geographic transects ending in SSAm and Canada, respectively. The latter shows a significant correlation with latitude for both founding (Slope -14.79; R^2

0.8055; P-value 0.0152) and expansion ages (Slope -11.71; R^2 0.9629; P-value 0.0005). These results partially resemble those obtained for Hg B2 in its North American subcontinent range. Congruent with its Andean center, the oldest haplotypes for C1c are found in NWSAm and Mexico (Supplementary_Table_ S3.11). The shortest genetic distances within Hg C1c (Supplementary_Table_S3.8) indicate the existence of three geographic centers: the first represented by the CSAm-CAm pair (0.571), the second by the SSAm-NWSam pair (0.630), and the third by the NWSAm-Mexico pair (0.644). This pattern could be explained by supposing NWSAm as the leading center that spread downward to Argentina and Chile and upward to Mexico, and a later center that, from Brazil, reached Central America, erasing the signal of the previous route from NWSAm to Mexico in this region. In this respect, it is worth mentioning that the Y-chromosome Q-M925, mainly Mesoamerican, has close phylogenetic ties with the Brazilian Q-Y26547 subclade (Colombo et al. 2022). This complex pattern is partially reflected in the PCoA plot (Fig. 4), which shows a latitudinal cline from NWSA to Canada passing through NESAm, with the unusual inclusion of SSAm in it, and an independent close relationship between Central America and Brazil.



For Hg C1c, the oldest regional ages for founding and expansion (Supplementary_Table_S3.14) are centered in NWSAm (≈ 34 kya / 26 kya). From this center, populations with younger ages extend along southern and northern geographic transects ending in SSAm and Canada, respectively. The latter shows a significant correlation with latitude for both founding (Slope -14.79; R^2 0.8055; P-value 0.0152) and expansion ages (Slope -11.71; R^2 0.9629; P-value 0.0005). These findings somewhat resemble those seen for Hg B2 across its North American range.

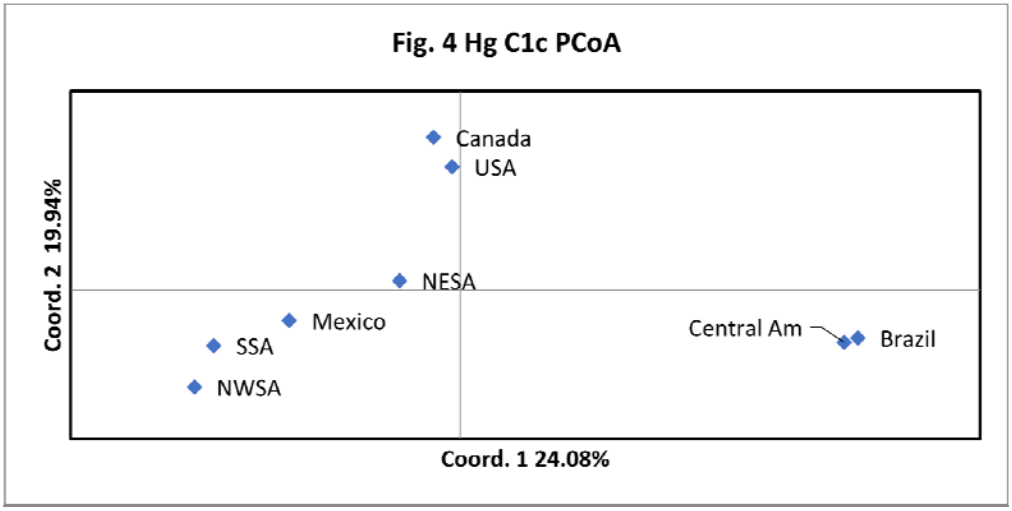
Congruent with its Andean origin, the oldest haplotypes for C1c are found in NWSAm and Mexico (Supplementary_Table_S3.11).

The shortest genetic distances within Hg C1c (Supplementary_Table_S3.8_ S3.8) indicate the existence of three geographic centers: the first represented by the CSAm-CAm pair (0.571), the second by the SSAm-NWSam pair (0.630),

and the third by the NWSAm-Mexico pair (0.644). This pattern could be explained by assuming NWSAm as the primary center that spread downward to Argentina and Chile and upward to Mexico, with a later center originating from Brazil that reached Central America, obscuring the previous route from NWSAm to Mexico. Notably, the Y-chromosome Q-M925, mainly Mesoamerican, has close phylogenetic ties with the Brazilian Q-Y26547 subclade (Colombo et al. 2022). This complex pattern

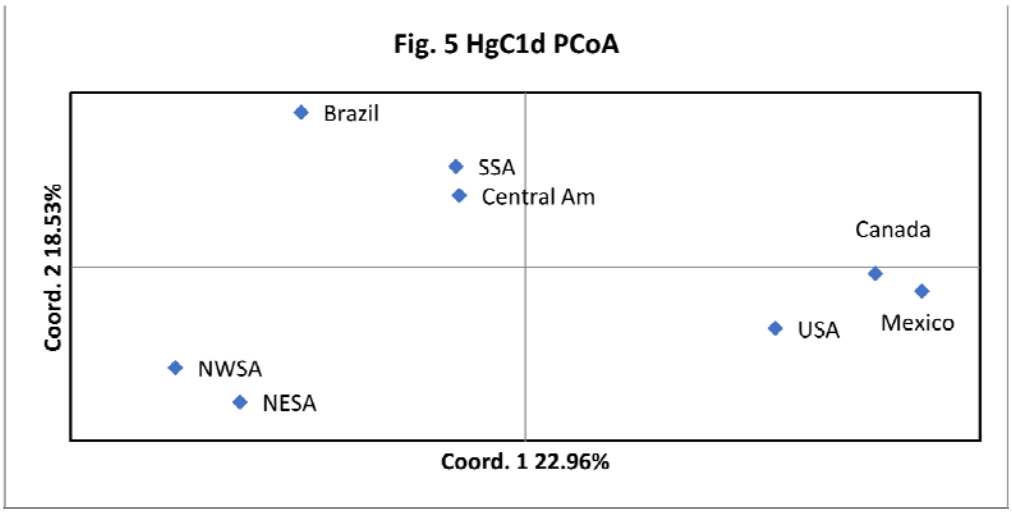
The oldest ages for the founding and expansion of haplogroup C1c (Hg C1c), as shown in Supplementary Table S3.14, are centered in Northwest South America (NWSAm), estimated around 34,000 to 26,000 years ago. From this central point, younger populations form geographic transects extending south to Southern South America (SSAm) and north to Canada. The data show a significant correlation with latitude for both founding (slope: -14.79; R^2: 0.8055; P-value: 0.0152) and expansion ages (slope: -11.71; R^2: 0.9629; P-value: 0.0005). These results resemble those observed for haplogroup B2 in its North American distribution. Consistent with its Andean origin, the oldest haplotypes of C1c are found in NWSAm and Mexico (as shown in Supplementary Table S3.11). Analysis of genetic distances within Hg C1c, detailed in Supplementary Table S3.8, reveals three geographic centers: the first is represented by the Central South America-Central America (CSAm-CAm)

pair (distance: 0.571), the second by the Southern South America-Northwest South America (SSAm-NWSAm) pair (distance: 0.630), and the third by the Northwest South America-Mexico (NWSAm-Mexico) pair (distance: 0.644). This pattern suggests NWSAm may be the original center of expansion, spreading southward to Argentina and Chile, and northward to Mexico. A later center, likely originating from Brazil, may have reached Central America, obscuring evidence of the earlier route that linked NWSAm to Mexico. Notably, the Y-chromosome Q-M925, primarily found in Mesoamerica, shows close phylogenetic relationships with the Brazilian Q-Y26547 subclade (Colombo et al. 2022). This complex pattern is partly demonstrated in the Principal Coordinates Analysis (PCoA) plot (Fig. 4), which displays a latitudinal gradient from NWSAm to Canada, passing through Northeast South America (NESAm), while also revealing the unexpected inclusion of SSAm. Additionally, there is a clear close relationship between Central America and Brazil.



Hg C1d, with the oldest founding and expansion ages (Supplemental_Table_S3.15) in CSAm and SSAm (approximately 38 kya / 33 kya), is the oldest C1 branch. In fact, it is the oldest Native American clade. Although not reaching statistical significance, C1d shows a geographic trend of decreasing population ages from North America to Central America (Supplemental_Table_S3.15). This pattern resembles the one observed for Hg A2. Additionally, it exhibits a significant latitudinal correlation from SSAm to Central America, with the oldest founding (Slope -16.65; R^2 0.7649; P-value 0.0415) and expansion ages (Slope -13.29; R^2 0.6985; P-value 0.0382) in southern South America. This geographic trend within the South American subcontinent mirrors the pattern seen in Hg B2. The oldest C1d haplotypes are found in CSAm and SSAm (Supplemental_Table_S3.12), supporting the idea of an ancestral southern South American center of expansion for this clade. For C1d, the closest genetic distances (Supplemental_Table_S3.9) are between the SSAm-CSAm pair (0.591) and the NWSAm-NESAm pair (0.592). The latter reflects the close genetic relationship between these regions, also seen in the C1b clade. The PCoA bi-dimensional representation of these distances (Fig. 5) shows a central group linking SSAm and CSAm with Central America, and two distant groups: one on the left joining NWSAm and NESAm, and another on the right grouping the northern North American populations and Mexico. The

independent and close genetic relationship between the South American regions and Central America resembles what was found for C1c, which was explained by a later expansion from Brazil to Central America, while the close genetic affinity between NWSAm and NESAm mirrors what was observed in C1b.



Haplogroup C4c

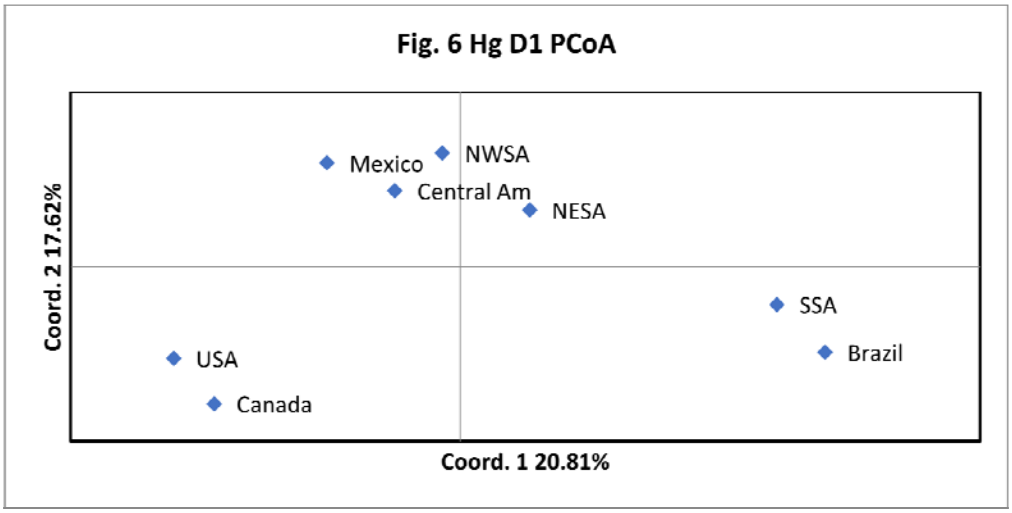
The founding and expansion ages for C4c are approximately 25 and 17,000 years ago, respectively (Table 2), but ages for its main branches vary significantly (Supplemental_Table_S3.19). The most developed branch, C4c1, has founding and expansion ages of about 16 and 12,000 years ago, both following the LGM. In contrast, the less common C4c2 sister branch has a founding age of 31 years ago, aligning more closely with other main Native American haplogroups. Still, its expansion age is very recent, at roughly 3,000

years ago. C4c is a minor haplogroup with a limited geographic distribution that, aside from a sporadic find in Colombia and an ancient sample from Brazil, Loca Do Suin_9100 years old (Ferraz et al. 2023), is confined to NNAm (Supplemental_Fig_S3). Therefore, the unusual results observed for this lineage may result from limited sampling and/or strong drift effects.

Haplogroup D1

For D1, the oldest founding ages (around 31,000 years ago) are found in SSAm and CSAm (Supplementary_Table_S4.5). The earliest expansion ages are also identified in these same regions but at different times (CSAm approximately 23,000 years ago; SSAm approximately 18,000 years ago). Regarding regional D1 founding ages, there is a significant latitudinal correlation from SSAm to Central America (slope -16.65; R^2 0.5431; P-value 0.0370), while a non-significant decreasing trend is observed from Canada to Central America (Supplementary_Table_S4.5). The oldest D1 haplotypes are primarily located in NWSAm and later in SSAm (Supplementary Table S4.4). The shortest genetic distances in D1 are between SSAm-CSAm (0.618), USA-Canada (0.667), and those involving NWSAm with SSAm (0.768) and with NESAm (0.759). A PCoA plot (Fig. 6) visually illustrates these genetic relationships, placing all Mesoamerican and northern South American populations in a central position, distinctly separated from two groups—one consisting of northern North

American populations and the other of southern South American populations, which are very distant from each other.



The D1 phylogeographic data described above closely resemble the patterns observed for haplogroups B2 and C1d.

Haplogroup D4h3a

For D4h3a, the oldest founding age (approximately 30,000 years ago) and expansion age (roughly 22,000 years ago) occurred in CSAm (Supplementary_Table_S4.8). Age comparisons between regions are only significant when the oldest expansion ages of SSAm and CSAm are compared to those of NNAm and NWSAm (Supplementary Table S4.9). When living separately, one can observe that NESAm is isolated; the oldest D4h3a haplotypes are found in NWSAm, Mexico, and consistently in Chile (Supplementary_Table_S4.7). It has been suggested that D4h3a indicates a

Pacific southward expansion (Perego et al. 2009). However, these results do not support that idea. It appears that D4h3a was more common in the past and was most frequent in eastern regions compared to today.

Haplogroup X2

Haplogroup X2a is regarded as a minor Native American haplogroup with a limited geographic range confined to northern North America (Perego et al. 2009). The estimated ages for the origin and spread of haplogroup X2a are approximately 29 years ago and 16 years ago, respectively (Supplementary_Table_S5.1), with the oldest haplotypes found in the X2a1 branch in samples from the North American Great Plains. These ages are notably older in North America than in Canada (Supplementary Table S5.3). However, the discovery of a divergent X2 haplotype in Canada (Perego et al. 2009), which may define a new Native American haplogroup called X2g (Supplemental_Fig_S5), is noteworthy. Although the current distribution of X2a, both ancient and modern, is concentrated in the northeastern part of North America (Perego et al. 2009), with sporadic cases in Mexico (Peñaloza-Espinosa et al. 2007; Bodner et al. 2021), its oldest known sample, the Kennewick Man- approximately 9000 years old and carrying a basal X2a haplotype- was excavated in northwestern Washington state (Rasmussen et al.

2015). This finding raises the possibility that the X2a clade may have originated in the northern Pacific region (Perego et al. 2009).

NRY haplogroups

On a global scale, two major parental lineages with Pan-American distribution have been identified (Q-CTS1780/Z780 and Q-M3), both within haplogroup Q, with the first occurring at lower frequencies everywhere compared to the second. Additionally, two minor lineages belonging to haplogroup C, with a subcontinental range (C-MPB373 and C-P39/Z30536), are considered Native American founder lineages. The southern C-MPB373 has been consistently reported in present-day Colombians and Equatorians (Karafet et al. 1999; Roewer et al. 2013; Mezzavilla et al. 2015; Villaescusa et al. 2021). The northern C-P39/Z30536 has been found in Canada and northern North America, reaching as far as Mexico (Perez-Benedico et al. 2016), showing a northern to southern gradient of decreasing diversity (Dulik et al. 2012). Although phylogenetically Q-CTS1780/Z780 is the oldest clade, the demographic expansion following the LGM mainly occurred among males within the Q-M3 lineage that branched off simultaneously, mainly in Mesoamerica (Q-M925) and South America (Q-M848), forming well-differentiated regional subclades (Bortolini et al. 2003; Battaglia et al. 2013; Roewer et al. 2013; Geppert et al. 2015; The Genographic Consortium et al.

2016; Grugni et al. 2019; Pinotti et al. 2019; Gómez et al. 2021; Villaescusa et al. 2021; Colombo et al. 2022; Kemp et al. 2007; Paz Sepúlveda et al. 2022). Some of these minor Q subclades are also noteworthy due to their geographic distributions and STR diversity. One such lineage is Q-MEH2, identified in northeast Siberian Koryaks. Its close relationship to the extinct Paleo-Eskimo Saqqaq (Rasmussen et al. 2010) suggests it could trace a recent migration from Siberia to America across the Bering Strait (Malyarchuk et al. 2011). However, Q-MEH2 has recently been found in Nahua populations from southern Mexico, and based on its STR variability, it appears to have arrived much earlier in Mexico than in northern America (Gómez et al. 2021). Another notable example is the Mexican Q-PV3 lineage, initially considered a minor branch of Q-L54, but its quite divergent haplotypic variability suggests it might represent another founding Native American lineage (Gómez et al. 2021). This subcontinental differentiation of male ancestries supports diverse demographic histories in North and South America (Bortolini et al. 2003). However, before drawing conclusions, it is necessary to examine what NRY ancient DNA analyses indicate, especially since post-Columbian admixture heavily impacted the Native American male genetic pool in both North (Malhi et al. 2007) and South America (Resque et al. 2016). The picture was similar. Although Q-CTS1780 might have been more common in the past across both

subcontinents (Posth et al. 2018; Grugni et al. 2019; Ferraz et al. 2023), Q-M3 was also the dominant NRY lineage in all ancient DNA data examined (Bisso-Machado and Fagundes 2021). Nonetheless, the detection of today's extinct lineages in the past (Posth et al. 2018; Colombo et al. 2022) supports the idea that strong founding and drift effects occurred in NRY Native American lineages. These findings do not align well with the hypothesis of a rapid and recent migratory wave from the north to explain the prehistoric colonization of South America.

DISCUSSION

The early arrival of Native uniparental lineages in the Americas, over 30 kya, as proposed here, renders the hypotheses of a prolonged (Hoffecker et al. 1993; Tamm et al. 2007) or brief (Pinotti et al. 2019) standstill in Beringia, a previous expansion of their ancestors into Asia (Wei et al. 2018; Sun et al. 2021; Ning et al. 2020), or the existence of a Pacific Coastal Route around 20 kya (Scheib et al. 2018; Davis and Madsen 2020; Li et al. 2023), unnecessary.

The archaeological evidence for humans in the Siberian Arctic around 30 kya (Pitulko et al. 2004) and possibly since 45 kya (Pitulko et al. 2017) supports the idea of an early entrance into America by its native ancestors. More challenging is gaining acceptance from archaeologists for human occupation in

the American continent before and around the LGM, but it looks pretty likely that this occurred around 16-18 kya at Monte Verde, Chile (Dillehay et al. 2015), at the Santa Elina site in Central Brazil during the LGM, at Chiquihuite Cave in Central Mexico about 30 kya (Ardelean et al. 2020), in the Colorado Plateau more than 36 kya (Rowe et al. 2022), at White Sands, New Mexico, during a stratigraphic record spanning from 24 kya to 17 kya (Holliday et al. 2025), and at Bluefish Caves, Canada, approximately 24 kya (Bourgeon et al. 2017).

Ancient genome studies have characterized the ancestral Native American population as a mixture of northern Eurasians and northern East Asians (Raghavan et al. 2014). The same dual contribution was previously suggested using NRY data (Karafet et al. 1999; Lell et al. 2002; Roewer et al. 2013), and a similar conclusion has been reached here based on mtDNA data. Genomic research indicates that the ancestral Native Americans arrived in the New World as an already admixed group (Moreno-Mayar et al. 2018). However, although coalescence ages do not show significant differences among haplogroups, the NRY phylogeny is more consistent with two successive waves: the first carrying C-MPB373 and Q-Z780 lineages, and the second comprising carriers of C-P39/Z30536 and Q-M3 lineages. Evidence for mtDNA is less conclusive, but the fact that the basal branches connecting haplogroups C and

D to their respective Asian roots are significantly shorter than those of A and B could support the idea that C and D arrived earlier into America. Another clue might be found in the Southern Cone of South America, the region farthest from the continent's entry point. Ancient and modern samples from this area show that haplogroups C and D were the first and predominant lineages reaching the region—still the most common today (Lalueza 1997; García-Bour et al. 2000; Moraga et al. 2000; Demarchi et al. 2001; Nakatsuka et al. 2020; Postillone et al. 2020). The dominance of haplogroups C and D has also been observed in Amazonian populations from Brazil (Merriwether and Ferrell 1996), suggesting an early southward migration. Furthermore, mtDNA diversity for haplogroups C1 and D1 increases from north to south, while North American populations exhibit higher diversity levels for haplogroups A2 and B2 (Lee and Merriwether 2015). In North America, haplogroups A, B, and X have expanded across populations in a clinal pattern, whereas there is no evidence of such spread for haplogroups C and D (Malhi et al. 2001). These patterns are consistent with two successive migration waves. However, they may also be explained by genetic drift, as samples from other regions show the absence of some founder mtDNA haplogroups. For example, the Kuna from Panama lack C and D (Batista et al. 1995), while the Ayoreo from Bolivia show no A or B haplogroups (Dornelles et al. 2004). Based on the distribution of two rare

mtDNA haplogroups, D4h3 and X2a, with the first spreading along the Pacific coast and the second entering via the ice-free corridor, two migratory waves from Beringia have been proposed (Perego et al. 2009). Nevertheless, for D4h3, the authors noted that its rarity in North America and higher frequency and variation in South America require further explanation (Perego et al. 2009).

Another hypothesis, based on genome studies, suggests that after arriving in North America, the native ancestral population split into two distinct branches known as South Native Americans (SNA) and North Native Americans (NNA) (Moreno-Mayar et al. 2018), or their approximate equivalents, Ancestry A (ANC-A) and Ancestry B (ANC-B), respectively (Scheib et al. 2018). While the northern ancestry is mostly limited to Canada and eastern North American regions, the southern ancestry is detectable in the oldest sampled genomic sequence, Anzick-1, which is approximately 12700 years old from Montana (Rasmussen et al. 2014), and in the Spirit Cave sample from Nevada, around 10700 years old (Moreno-Mayar et al. 2018), extending southward to Mesoamerica and South America (Moreno-Mayar et al. 2018). There are single-parental markers that share the geographic range of NNA genomic ancestry, such as mtDNA haplogroups X2a (Brown et al. 1998) and C4c (Kashani et al. 2012), as well as NRY lineages C-P39 (Pinotti et al. 2019), Q-Y4276 (Grugni

et al. 2019). The situation becomes much more complex when explaining the transcontinental distribution of the southern component of the Native American genome. The initial dual explanation involving southward Pacific coastal expansion—where successive populations could be seen as mixtures of SNA and NNA components in varying proportions (Scheib et al. 2018)—was proven insufficient (Posth et al. 2018). First, the uniqueness of the northernmost SNA component, represented by the Clovis-associated Anzick-1 sample (SNA1), could not account for the genomic makeup of many South American populations, leading to the need to add a new, dominant non-Clovis SNA2 component, partly represented by Spirit Cave ancestry, to better explain the genomes of many southern populations. Approaching Mesoamerica, the Mexican Mixe population required gene flow from an unsampled population for accurate representation (Moreno-Mayar et al. 2018). Furthermore, several undiscovered genomic ancestries needed to be included to model the structure of various South American regional populations properly. An interesting case is the presence of a divergent ancestry related to Australasian people found in Amazonian tribes such as Surui, Karitiana, and Xavante (Raghavan et al. 2015; Skoglund et al. 2015). This component, called "population Y," was confirmed in ancient Brazilian samples like Sumidouro-5, approximately 10000 years old (Moreno-Mayar et al. 2018). At least one

mtDNA haplotype, likely from haplogroup B—whose southernmost lineage aligns well with the Australasian ancestry—was necessary to accompany this component. However, identifying the ancestral Asian population from which it entered the New World remains challenging. Basic lineages of haplogroup B appeared in eastern Asia around 40,000 years ago, as shown by the Chinese Tianyuan sample (Fu et al. 2013). The Australasian ancestry shared by ancient South American samples and Tianyuan is also notable (Yang et al. 2017), suggesting it could have arrived with populations deeply rooted in eastern Asia with ancient Australasian ties, or with carriers of mtDNA haplogroup B from central Eurasia. The Isthmus-Colombian region also showed genomic complexity, with a Pleistocene ancestry reaching Panamanians and remaining confined to the Isthmus (Capodiferro et al. 2021). Hunter-gatherers from the Colombian Altiplano also carried an unknown basal lineage from the initial South American genomic radiation (Krettek et al. 2025). In northwest South America, a previously unrecognized ancestry was distinctive of northern Ecuador and Colombian populations (Barbieri et al. 2019), while Native Peruvians from the Lake Titicaca area showed a Siberian component also found in ancient North Americans like Anzick-1, Kennewick, and Saqqaq (Lindo et al. 2018). The complex ancestries uncovered in South America have led some scholars to caution against oversimplifying the rapid post-glacial colonization

model, suggesting that a northward expansion from South America to explain the Pacific migration wave would require a more intricate history (Posth et al. 2018). Others propose that the dispersal pattern of SNA south of the continent's ice sheets involved complex admixture events among earlier populations (Moreno-Mayar et al. 2018). The uniparental markers analyzed in this study offer an alternative hypothesis, potentially integrating all genomic data from a different perspective.

If we accept that Asian populations gave rise to Native Americans before 30 kya, then the story of human settlement in America can be viewed differently. It is known that since their arrival, the climate gradually worsened, covering Arctic and Antarctic regions with ice (Clark et al. 2009), clearing the Amazon and Central American rainforests (Häggi et al. 2017), and transforming most of the land into forests and dry steppes (University of Geneva, Switzerland et al. 2001). During this tough period, humans had to survive in small, isolated groups and adapt to different glacial refugia, leaving few archaeological traces. When the climate improved, these groups grew in number and expanded into new, now habitable areas. Unlike the rapid southern wave of colonization, the American continent was not empty of humans. The post-LGM colonization was not a single or multiple waves spreading from northern North America to settle Mesoamerica and then South America. Instead, it involved more or less

simultaneous radiations from various regions, most of which, based on uniparental markers, are centered in South America. One major expansion center for mtDNA is the northwest Colombian-Isthmus area, where younger haplogroup A2 and C1b clines are seen stretching north and south. A similar distribution of A2 frequencies has also been observed in this region (Keyeux et al. 2002). Another potential core is in the Andean region, where mtDNA haplogroup C1c shows its oldest origins and expansion ages, with younger clines extending toward Canada and down to SSAm. This pattern mirrors that of haplogroup B, which expanded from Chile and the Andean region into Mesoamerica. However, it appears that at least part of the B2 cline results from displacement by other mtDNA lineages in North America, as B was present early on in northeast Asia as an ancestral B lineage (Fu et al. 2013) and in Alaska as a basal B2 lineage (Tackney et al. 2015), before nearly disappearing from both areas. The highest frequencies and genetic diversity of haplogroup B2 are found in the Andean Altiplano (Gayà-Vidal et al. 2011; Taboada-Echalar et al. 2013), the Bolivian Piedmont (Corella et al. 2007), and the Argentinian Gran Chaco (Demarchi et al. 2001). Although the classic explanation for the B2 cline suggests descent from California (Taboada-Echalar et al. 2013), South American origins have also been proposed, from the Peruvian Andes (Arencibia et al. 2025) or the Chilean Southern Cone (Bravi et al. 2024). Similarly, the C1d

haplogroup, like B2, shows its most ancient origins and expansion in the southern Cone, spreading northward with younger ages toward Central America, indicating a significant geographic pattern. In North America, some lineages of C1d show an expansion history that ends in Central America, paralleling trends observed in haplogroup A2 sub-lineages. C1a (Tamm et al. 2007), a northeast Asiatic branch of C1, along with minor European sub-clades C1e (Ebenesersdóttir et al. 2011) and C1f (Der Sarkissian et al. 2014), suggest a later reverse migration from America (Tamm et al. 2007). Using the evolutionary rate proposed here, the founding age of C1a in Asia is about 26.02 kya (95% CI: 18.58-33.49 kya), signaling a return to Asia during worsening weather conditions. Its spread across Asia around 13.9 kya (95% CI: 6.46-21.37 kya) points to a Mesolithic migration, similar to the pattern seen in the northeast European C1f clade (Der Sarkissian et al. 2014). This early American retro-migration might also be supported by the phylogeny and phylogeography of the Y-chromosome haplogroups C-L1373 (Pinotti et al. 2019; Colombo et al. 2022) and Q-L54 (Grugni et al. 2019; Pinotti et al. 2019).

Another mtDNA haplogroup indicating the southern Cone region as a key center of expansion is D1, which shows a significant negative correlation from this area to Central America. Its mean coalescent ages are younger as you move upward, although there is a longitudinal bias leaning toward central and

eastern South American regions. D1 is most common in the eastern parts of the Andean region, with ancient regional clades in Central Argentina (García et al. 2012) and the southern Cone (Bodner et al. 2012). Similarly, for A2 and C1d, a southward expansion from Canada to Central America is observed for D1. These expansion patterns might explain the B2 retraction seen in North America. The idea that mtDNA haplogroup D4h3a supports a Pacific coastal route for peopling of the Americas (Perego et al. 2009), later backed by genomic studies (Moreno-Mayar et al. 2018), is not supported by the evidence here. The earliest ages for the founding and expansion of D4h3a are found in SSAm and CSAm, and are significantly older than those in NWSAm and NNAm (Supplementary_Table_S4.9_ S4.9). Additionally, although D4h3a is absent today, it has been found in ancient samples from Alaska, Shuká-Káa, 10.3 kya (Lindo et al. 2017), from British Columbia, Canada, 939, 6.5 kya (Lindo et al. 2017), and Montana, Anzick 1, 12.6 kya (Rasmussen et al. 2014). It was also detected in ancient remains from the Brazilian Amazon, Lagoa Santa, 10 kya (Moreno-Mayar et al. 2018). This pattern closely resembles that of B2. Therefore, rather than modeling a southward Pacific Coastal expansion, both markers seem to be remnants of a broader past occupation that was displaced by more recent human migrations.

A specific area in the Amazon Basin (CSAm) deserves special mention. It participated in all the mtDNA radiation centers discussed earlier and is known as a major reservoir of deep mtDNA lineages (De Souza et al. 2025; Di Corcia et al. 2017). It stands out for the ancient presence of very deep-rooted Y-chromosome lineages such as C-L1373* (Colombo et al. 2022) and for the detection of lineages that were previously thought to be unique to the northern subcontinent, like Q-Y4303* (Grugni et al. 2019). This genetic diversity is also supported by whole-genome studies on ancient specimens (Moreno-Mayar et al. 2018) and current populations (Ribeiro-Dos-Santos et al. 2020; Capodiferro et al. 2023). This genetic landscape aligns with the region's linguistic richness. Notably, out of 71 identified linguistic isolates across the continent, over 45% are in Brazil, compared to 15% in North and Mesoamerica combined (Blench and Cb). Future archaeological findings will likely uncover human settlements much older than currently recognized. Furthermore, the differences in genetic and linguistic diversity between the northern (NNA) and southern (SNA) American branches make the idea of simultaneous colonization of both subcontinents from a northward expansion unlikely.

MATERIAL AND METHODS:

Material

For the mtDNA phylogenetic and phylogeographic analyses, I searched for partial and complete mitogenomes in the NCBI GenBank, www.ncbi.nlm.nih.gov/genbank/ ; Mitomap, www.mitomap.org/MITOMAP ; EMPOP, https://empop.online ; Ian Logan 2023, www.ianlogan.co.uk/sequences_by_group/haplogroup_select.htm ; Genome Warehouse (GWH) public repository, https://bigd.big.ac.cn/gwh ; and AmtDB, www.http://amtdb.org . Additionally, for phylogeographic analyses only, I also searched YFull and MTree. The NRY phylogenetic and phylogeographic analyses were based on the ISOGG database, http://www.isogg.org/tree/ ; YFull YTree v13.03.00; YTree; and data compiled by other authors (Wei et al. 2018; Grugni et al. 2019; Pinotti et al. 2019; Gómez et al. 2021; Colombo et al. 2022; Paz Sepúlveda et al. 2022). For brevity, the Y-chromosome lineages in the text are referred to by the letter of their basic haplogroup and their terminal mutation.

Phylogeny

Maximum parsimony intraspecific phylogenetic trees (Bandelt et al. 1999) were constructed manually, and haplogroup nomenclature followed the PhyloTree database Building 17, http://www.phylotree.org. Haplogroup

classification was confirmed using HaploGrep 3 3.2.1, https://haplogrep.i-med.ac.at (Weissensteiner et al. 2016). Newly identified subgroups not classified in PhyloTree were provisionally named by the first letter and digit of their primary haplogroup, followed by an asterisk and consecutive numbers. Both labels are shown on the respective tree when a more formal nomenclature is available in YFullMTree.

Evolutionary rates

It is well known that the mtDNA evolutionary rate experiences a time-dependent effect, slowing down as we go back in time (Henn et al. 2008). The main reason for this effect has been linked to differences in population size, with the smallest populations during Paleolithic times and exponential growth in recent times (Cabrera 2021a). To account for this anomaly, I used an estimated Paleolithic mitogenome substitution rate of 1.5 x 10^-8 (95% CI: 0.42 - 9.07 x 10^-8) per site per year (assuming a mtDNA genome length of 16,500 base pairs), meaning one substitution occurs roughly every 4,040 years (Cabrera 2024), to estimate haplogroup coalescent ages for the early prehistoric settlement of the Americas. Using a similar approach, a proposed mean evolutionary rate of 0.47 x 10^-9 (95% CI: 0.21 – 0.95 x 10^-9) per site per year was used to calculate coalescent ages of the Y-chromosome (Cabrera 2024).

Ages

I have used three approaches to estimate haplogroup coalescent ages: 1) applying statistics rho (Forster et al. 1996) and Sigma (Saillard et al. 2000) on individuals within haplogroups, along with the evolutionary rates mentioned above; 2) utilizing clades within main haplogroups to calculate a pooled mean, then applying normal averaging and standard error statistics for mutation rates across haplogroups; 3) since the sample sizes of these clades vary significantly, I calculated weighted averages and weighted standard errors to apply with the same normal statistics; 4) a common discussion in genetics is that haplotypes at low frequency might become much more common within a population, although this is generally an exception; typically, rare haplotypes—those at the tails of a distribution—are likely to go extinct due to genetic drift, while haplotypes near the mean tend to increase in frequency. Given that the Native American founding haplogroups have experienced genetic drift over time, I used the most divergent lineages within them as proxies for their past means to estimate coalescence ages (Cabrera 2022; Cabrera 2021a).

Phylogeography

For phylogeographic purposes, the American continent has been divided into seven geographic regions: 1) North Northern America (NNAm), including

Alaska and Canada; 2) North America, covering the entire United States (Nam); 3) Mesoamerica, comprising Mexico, Belize, Guatemala, El Salvador, Honduras, and Nicaragua (Mam); 4) Northeastern South America or Isthmus-Colombian area, including Costa Rica, Panama, and Colombia; 5) Northwestern South America (NWSAm), also known as the Andean region, including Ecuador, Peru, and Bolivia; 6) Central South America (CSAm), including Brazil, Paraguay, and Uruguay; 7) Southern South America (SSAm), including Argentina and Brazil. Additionally, within these regions, distinctions are sometimes made between inland and coastal Pacific and Atlantic areas, as well as between Highland Andes and Amazonian zones. To support the phylogenetic and phylogeographic analyses, I have implemented some straightforward algorithms described below.

Genetic distances

In addition to the molecular distance based on the number of mutations between two homologous DNA sequences (FST), I have implemented a phylogeographic distance (DXY) based on genetic affinity (IXY) between regions X and Y. This distance is measured by the frequency with which they form sister branches within a haplogroup tree (nXY) relative to how often each region participates as a sister branch within the measured haplogroup (nX and nY).

$$I_{xy} = \frac{2n_{xy}}{nx + ny}$$

Then D_{XY} is calculated as $1 - I_{XY}$.

Additionally, when searching for sister branches between X and Y regions within a haplogroup, I also observed cases where one sequence from one of the involved regions served as a direct outgroup to the pair, indicating it as ancestral relative to the other region, which was considered derived. The significance of this relationship was tested using observed-expected chi-square statistics.

Additionally, for phylogeographic purposes, I have examined each major Native American founder haplogroup to identify the regions where their oldest sub-haplogroups are found and the areas from which they initially expanded.

Finally, to identify the Asian centers where the ancestors of the major American founder clades may have originated, I searched for the most ancient Asian sequences (less mutated) that are from the Asian-American shared phylogenetic nodes and are found in these regions.

Other statistics

Analysis of molecular variance (AMOVA), correlation/linear regression, and graphical Principal Coordinates Analysis (PCoA) were performed using the

GenAlEx 6.5 software (Peakall and Smouse 2012). Descriptive normal statistics, 95% confidence intervals for means, and two-tailed t-tests were conducted with GraphPad https://www.graphpad.com/quickcalcs/. Weighted means and standard errors were calculated using the Weighted calculator (https://calculatodo.com).

False positive correction

Since I performed multiple statistical tests for each founder haplogroup, I applied a Bonferroni p-value adjustment. However, because the Bonferroni correction is very conservative, I also used the Benjamin and Hochberg 1995 (Benjamini and Hochberg 1995) false discovery rate (FDR) method as a less restrictive alternative.

DATA ACCESS

All the sequences and haplotypes used in this study are available in the public domain. Databases and references are detailed in the text.

COMPETING INTEREST STATEMENT

The author declares no conflicts of interest regarding this work.

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TITLE:

What uniparental genetic markers tell us about the prehistoric human colonization of the Americas

INTRODUCTION:

The interpretation of the prehistoric peopling of the Americas has been a multidisciplinary enterprise (Becerra-Valdivia and Higham 2020; Fiedel 2000). From a molecular genetics perspective, the first foundations of these interpretations were based on the information provided by the uniparentally inherited mitochondrial DNA (mtDNA) and the non-recombining portion of the Y-chromosome (NRY) on extant populations (Schurr 2004; Karafet et al. 1999). As ancient DNA (aDNA) samples are a mixture of endogenous aDNA and

contaminating DNA, at first, its results on human samples were questioned. Fortunately, new sequencing technologies have managed to overcome this problem (Palencia-Madrid and De Pancorbo 2015), and current aDNA-based analyses have added an invaluable timing perspective to these interpretations (Bisso-Machado and Fagundes 2021). Later, technological improvements have made it possible to sequence entire genomes in both present-day and fossil individuals. Analyses of these genomes have revealed extinct ancestries and genetically well-differentiated ancestries from the past that, admixed in different proportions, persist in current Native American populations along the American continent. This new information has served to improve previous hypotheses and to formulate new ones (Willerslev and Meltzer 2021).

Thus, some conclusions have achieved general acceptance: 1) The Asiatic origin of the Native American ancestors; 2) The first migration, in one or several waves, occurred as a rapid movement towards the south of the double continent; 3) The initial low population density led to strong genetic differentiation geographically structured; 4) Subsequent migrations reshaped the genetic landscape of North and South America. Important for the first was the entrance from Beringia of several migratory waves that originated the settlement of Paleo- and Neo-Eskimos; 5) Time comparisons of population

genetic profiles since post-Neolithic to present-day have uncovered an important genetic continuity (although see (Llamas et al. 2016)).

However, other propositions are still under debate as: 1) the precise Asian origin of the Native American ancestors; 2) the routes followed by these pioneers as they expanded throughout the Americas; and, perhaps, the most contentious question, 3) when these first spreads occurred? Although the most recent studies based on complete genomes favor a post-Glacial dispersal (Moreno-Mayar et al. 2018), with or without a prolonged standstill in Beringia (Faught 2017), the first works with mitochondrial DNA favored an early entry, around 30 kya before the Last Glacial Maximum (LGM), avoiding the problem of having to cross the ice sheets of North America (Bonatto and Salzano 1997; Torroni et al. 1994).

These age discrepancies are due to the molecular evolutionary rates used in each case. Unfortunately, the rate of molecular evolution is not a constant; it depends on several parameters, one of which, the effective population size (Ne), has changed dramatically since the Paleolithic to present-day (Cabrera 2020). Therefore, similar to radiocarbon dating, molecular rates need to be calibrated to obtain more accurate ages. One way to do this is by using ancient genomes and/or mitogenomes, but in the case of the American colonization, all the extracted aDNA postdates the LGM (Bisso-Machado and Fagundes

2021). The option chosen here is to use evolutionary rates calibrated with Paleolithic fossils to date Paleolithic events (Cabrera 2024).

Despite their unquestionable utility, uniparental markers are barely mentioned in whole-genome studies (Gusareva et al. 2025). But see (Moreno-Mayar et al. 2018; Krettek et al. 2025). The underlying reason for this abandonment is that these markers only offer monogenic insights, and that only the set of all genes provides congruent results. In my opinion, this assumption is flawed. The main cause of genetic differentiation used to estimate phylogenies and genetic distances between populations or species is isolation. Isolation affects whole genomes and does not discriminate by sex. Populations differentiate genetically, due to the loss of previous polymorphisms, by fixation or extinction, and the accumulation of new polymorphisms by mutation in a time-dependent way. Thus, any DNA fragment, including those coding for uniparental markers, should be valid to realize genetic comparisons whenever they have accumulated enough genetic differentiation. The generalized use of DNA barcoding in molecular taxonomy (Hubert and Hanner 2015) validates my previous assertion. Isolation is broken by migration/introgression, affecting uniparental markers and autosomes differently. Regardless of whether uniparental markers persist only in their original populations or whether both endure in the recipient population, their coalescence node—that is, the

distance to their most recent common ancestor—will remain unchanged. However, for mixed autosomes, if the introgressed fragments are not thoroughly masked, they will influence the genetic distances obtained between the populations/species involved. A paradigmatic example of that is the case of the controversial phylogenetic relationships obtained between modern humans and their closest relatives, Neanderthals and Denisovans. While mtDNA and NRY sequences showed modern humans and Neanderthals as sister lineages (Posth et al. 2017; Petr et al. 2020), autosomes joined Denisovan and Neanderthal as the closest pair, leaving modern humans as an out-group (Posth et al. 2017). Contrary to the widespread acceptance that the true relationship between hominids is that obtained with the autosomes, I have suggested that the valid phylogeny is the one deduced from the uniparental markers, while the one obtained from the autosomes is the result of later introgressions between the three groups of hominins (Cabrera 2021b).

Taking into account the above-mentioned particularities of the uniparental markers, in this work, I perform a thorough reanalysis of all published mitogenomes and the most recently published Y-chromosome results, using phylogenetic and phylogeographic tools, to reconsider and shed new light on the hypotheses formulated to explain the prehistoric colonization of the New World by Asian peoples. The main conclusions obtained are: 1) that the first

migration involved at least two Asian populations with most probable central and eastern Asian origins; 2) that this wave(s) occurred before 30 Kya; 3) that those pioneer colonizers did not stop in Beringia but kept trekking across the Americas leaving few genetic traces in past and present-day native American populations; 4) That after the LGM, small and relatively isolated resilient groups radiated from different areas to occupy new exploitable territories both in North and South America; 5) that these secondary expansive waves occurred first in southern- an meso-America than in northern North America; and 6) that gene flow existed among geographically adjacent groups.

RESULTS

All the phylogenetic and phylogeographic mtDNA results described below have been obtained from the trees presented in the supplementary figures (Suplemental_Fig_S1.xls (HgA2), Fig_S2.xls (HGB2), Fig_S3.xls (HgC1), Fig_S4.xls (HgD), and Fig_S5.xls (HgX2), and all the NRY results described below have been obtained from the trees already published by other authors (Wei et al. 2018; Grugni et al. 2019; Pinotti et al. 2019; Villaescusa et al. 2021; Colombo et al. 2022; Paz Sepúlveda et al. 2022).

The most probable origin of the American Asian ancestors

The main Native American mtDNA haplogroup founders can be divided into two groups: 1) those belonging to macro-Haplogroup M (C1, C4c, D1, D2a1, D4e1b, and D4h3a), and 2) those that belong to macro-Haplogroup N (A2, B2, and X2a). Curiously, the same grouping occurs when we look at the number of mutations accumulated in their basal stems that link them to the common nodes with Asian lineages. While C1, C4c, D1, D2a1 and D4h3a have two mutations, and D4e1b only one; A2 and B2 have five mutations and X2a four mutations (Table 1).

Table 1. Mutations on American basal branches to their common nodes with Asia

American haplogroup	**Mutations**	**Coalescent Age (Kya)**	**Asian haplogroup**	**Asian region**			
A2	5	20,2	A-a1	NW China (Xinjiang)			
A10a	1	4,04	A10	Western Siberia			

B2	5	20,2	B4b1a	Altai			
C1	2	8,08	C4	Eastern Asia			
C4c	2	8,08	C4a'b'c	Eastern Asia			
D1	2	8,08	D4	Eastern Asia			
D4e1b	1	4,04	D4e1	Eastern Asia			
D2a1	2	8,08	D2a'b	Northeastern Siberia			
D4h3a	2	8,08	D4h3	NW China (Xinjiang)			
X2a	4	16,16	X2a'j	Tajikistan			

An unpaired t-test shows that the difference between groups is highly significant (t = 7.2164; df = 5; two-tailed P value = 0.0008). Furthermore, it is well known that macro-Haplogroup M is dominant in southern and eastern

Asia (Marrero et al. 2016), while macro-Haplogroup N is more frequent in western Eurasia (Fregel et al. 2015). Consequently, the same occurs with the Asian ancestral sequences/haplogroups that show the shortest distances with the Native American haplogroups. It has already been demonstrated that the closest relatives for the macro-Haplogroup M American lineages are found in eastern Asia/southeastern Siberia (Starikovskaya et al. 1998; Derenko et al. 2010). Some further commentaries are pertinent for these lineages: Hg C1 comprises four common and well spread sub lineages, three of them (C1b, C1c, C1d) practicably exclusive of the Americas and one (C1a) with a wide Asian range, and several rare clades/isolates, the most ancient of which is represented by two identical sequences from Brazil (Simão et al. 2021) and Paraguay (Avila et al. 2019) that carries twelve mutations in its basal stem (Supplementary_Fig_S3.xls). Curiously, the other isolates have been detected in northwestern Eurasia instead of East Asia: C1e in Iceland (Ebenesersdóttir et al. 2011), western Russia (Järve et al. 2019), and western China (Wang et al. 2021); C1f in Tajikistan (Peng et al. 2018) and C1g in Mesolithic remains from western Russia (Der Sarkissian et al. 2014). As the oldest clades are found in the New World, the most parsimonious conclusion is that all the C1 branches found in Eurasia were the result of retro migrations from America. The oldest Native American samples harboring C1b lineages are USR1 from Alaska

(Tackney et al. 2015) and I11974 from Chile (Posth et al. 2018), both around 12,000 years old; for C1c it is MW291667 from Argentina (Roca-Rada et al. 2021), approximately 8,500 years old; and for C1d it is Lapa01, 9,600 years old, from Brazil (Posth et al. 2018). Haplogroup D1 has the oldest samples in Yucatan, Mexico, represented by a Late Pleistocene skeleton (HN5/48) around 12 ky old (Chatters et al. 2014), in Spirit Cave, Nevada, around 11 kya (Moreno-Mayar et al. 2018), an approximately 10 ky old LLP.S2.E1 sample from Argentina (Roca-Rada et al. 2021), and the Sumidouro4, 9,645 years old, sample from Brazil (Moreno-Mayar et al. 2018). Until recently, the American haplogroup D4h3a was separated from its Asian root by six mutations, but recent aDNA studies (Mao et al. 2021) have rescued a Chinese sequence that shares four of the six basal substitutions with the American clade (Supplementary_Fig_S4.xls). The remains, from which this sequence was extracted, were unearthed at the Amur region, in northeastern China, and are around 14,000 years old. The oldest samples harboring D4h3a sequences in the New World are the 12,600-year-old Anzick1 from Montana (Rasmussen et al. 2014), the 10,300-year-old Shuká Káa from Alaska (Lindo et al. 2017), and the 10,400-year-old specimens from Lagoa Santa in Brazil (Moreno-Mayar et al. 2018).

In relation to the macro-Haplogroup N, for the New World haplogroup A2, the closest Asian sequences are from haplogroup A-a1 (BZL_M16A, BZL_M16B) and belong to historic samples from Xinjiang, western China (Wang et al. 2021). For haplogroup B2, it is AY519494, included in the Asian haplogroup B4b (Supplementary_Fig_S2.xls), being the Altai region as its geographic origin (Starikovskaya et al. 2005). Finally, for the American haplogroup X2a, the closest Asian relatives are into haplogroup X2j, that groups samples from Egypt and Europe (Supplementary_Fig_S5.xls) but, for geographic proximity, I consider as the closest Asian sequence a partial one (sample 40) from Tajikistan (Ovchinnikov et al. 2014), as it carries 16179 and 16357 substitutions that are terminal mutations in the Egyptian X2j (Kujanová et al. 2009) mitogenomes (Supplementary_Fig_S5.xls). For haplogroups A2 and B2 the most ancient sequences, around 14,000 years old, were extracted from human coprolites found in Paisley Caves, Oregon (Gilbert et al. 2008), next oldest for A2 is Lapa15 (around 8,700 years old) from Lapa do Santo, Brazil (Posth et al. 2018), and for B2 is USR2 (approximately 12,000 years old) from Upward Sun River site in Alaska (Tackney et al. 2015). The most ancient Native American sample with an X2a haplotype is the Kennewick Man from Washington, which is around 9,000 years old (Rasmussen et al. 2015).

A way to reconcile the close arrivals of the Asian ancestors carrying the Native American M and N lineages with their significant differences in the number of mutations accumulated on their basal stems is to assume that the route followed across Asia of the N carriers was longer and slower than that of the M carriers.

Finally, if we compare the number of lineages carried by the M and N groups to their respective population sizes upon arrival in America, we could estimate that the contribution of the eastern Asian contingent was 62.5% and that of western Eurasia 37.5%.

The NRY does not show American colonization patterns as clear as those of mtDNA, but using its phylogeny and phylogeography in the Americas and Asia I have considered the ancestors of the American clades C-MPB373, that has its closest haplotypes in the eastern Asian outgroup clade C-L1373 (Pinotti et al. 2019; Sun et al. 2021), QF746 that includes the Q-YP1475 subclade represented by the Saqqaq ancient sample (Rasmussen et al. 2010) and has as sister subclade Q-B143 represented by northeastern Siberian Koryaks (Karmin et al. 2015), and Q-FT9713, that has its closest sequences in Mongolia and northeastern China (https://www.yfull.com/tree/Q) as of eastern Asian origin, while C-P39/Z30536, having as outgroup the Central Asian subclade C-F1756 (Wei et al. 2018), Q-Z780, that includes the Native American ancient

sample Anzick1 (Rasmussen et al. 2014) and has Q-L330 of Central Asia ascription (Dulik et al. 2012; Flegontov et al. 2019) as sister clade, Q-M3, that has as out group Q-L804 with a northern European geographic range (Hallast et al. 2015), and the rare Q-Y1150 and Q-M378 (Grugni et al. 2019) as of western Eurasian origin. Unlike mtDNA, it appears that the vast majority of the American male lineages had their ancestors in western Eurasia. It is interesting to note that the C-MPB373 South American lineage is phylogenetically an out-group of C-F1699 that comprises numerous western Eurasian lineages, including also the Canadian C-P39/Z30536 branch (Pinotti et al. 2019). Also of interest is that the most abundant American clade, Q-M3 (Colombo et al. 2022), has as sister branch Q-L804, which is specific to northern Europe (Grugni et al. 2019). The oldest sample belonging to the eastern group of lineages is Lapa01, from Brazil, which is approximately 8,840 years old (Posth et al. 2018). For the western group, Anzick1 from Montana with an approximate age of 12,600 years represents the oldest North American sample (Grugni et al. 2019), whereas in South America, the oldest sample (I11974) was found in Los Rieles, Chile, being approximately 10,500 years old (Posth et al. 2018).

The global ages of the first arrival and expansions of the Native American parental ancestors

A global average of the different mean mutational data, calculated for each American mitochondrial haplogroup using different approaches (Table 2), give mean mutational values of 7.11 (95% CI: 6.34 – 7.88) substitutions at the arrival in America of the founding lineages, and a mean of 4.42 (95%: 3.98 – 4.85) substitutions for their initial expansions into the American continent, which, in turn, produce mean ages of 28.72 (95%: 25.61 – 31.84) kya and of 17.86 (95% CI: 16.08 – 19.59) kya for the founding and expansion events respectively.

Table 2. Mutational means and coalescent mean ages for the main American mtDNA haplogroups

HG	**Phylogeny**	**Mean (Age Kya)**	**Type**	**N**	**95% CI: Mutation/Age(Kya)**			
A2	Founding	7.55 (30,50)	Normal	1119 (Indiv)	7.37-7.73 / 29,77-31,23			
	Expansi	4.63	"	1083	4.46-4.79 / 18,02-			

	on	(18,70)		(Indiv)	19,35			
	Foundi ng	7.25 (29,29)	Weight ed	118 (Clades)	6.62-7.88 / 26,74-31,83			
	Expansi on	4.35 (17,57)	"	118 (Clades)	4.04-4.66 / 16,32-18,83			
	Coalesc ent	9.13 (36,88)	Max (19 nt)	16 (Branche s)	8.46-9.79 / 34,18-39,55			
B2	Foundi ng	6.68 (26,99)	Normal	1081 (Indiv)	6.51-6.85 / 25,30-27,68			
	Expansi on	4.68 (18.90)	"	1070 (Indiv)	4.51-4.85 / 18,22-19,00			
	Foundi ng	6.62 (26,75)	Weight ed	80 (Clades)	6.21-7.03 / 25,09-28,40			
	Expansi	4.69	"	80	4.28-5.11 / 17,29-			

	on	(18,95)		(Clades)	20,64			
	Coalescent	6.42 (25,93)	Max (16 nt)	14 (Branches)	5.12-7.72 / 20,68-31,17			
C1	Founding	6.50 (26,26)	Normal	887 (Indiv)	6.33-6.67 / 25.57-26,95			
	Expansion	4.36 (17,61)	"	873 (indiv)	4.17-4.55 / 16,84-18,38			
	Founding	6.33 (25,57)	Weighted	138 Clades)	6.05-6.61 / 24,44-26,71			
	Expansion	4.01 (16,20)	"	138 (Clades)	3.71-4.31 / 14,99-17,41			
	Coalescent	6.22 (25,13)	Max (16 nt)	42(Branches)	5.54-6.90 /22,38-27,88			
C4c	Foundi	6.32	Normal	34 (Indiv)	5.74-6.91 /23,19-			

	ng	(25,53)			27,92			
	Expansion	4.32 (17,45)	"	34 (Indiv)	3.74-4.91 / 15,11-19,84			
	Founding	3.48 (14,06)	Weighted	8 (Clades)	2.85-4.11 /11,51-16,60			
	Expansion	1.30 (5,25)	"	8 (Clades)	0.67-1.93 /2,71-7,80			
	Coalescent	4.71 (19,03)	Max (9 nt)	3 (Branches)	2.47-6.96 /9,98-28,12			
D1	Founding	7.00 (28,28)	Normal	496 (Indiv)	6.73-7.27 / 27,19-29,37			
	Expansion	4.97 (20,08)	"	463 (Indiv)	4.68-5.27 / 18,91-21,29			
	Foundi	7.02	Weight	58	6.53-7.51/26,38-			

	ng	(28,36)	ed	(Clades)	30,34			
	Expansion	4.97 (20,08)	"	58 (Clades)	4.48-5.46 / 18,10-22,06			
	Coalescent	7.14 (28,86)	Max (16 nt)	28(Branches)	6.67-7.62 / 26,93-30,79			
D4h3a	Founding	6.20 (25,05)	Normal	80 (Indiv)	5.57-6.83 / 22,50-27,59			
	Expansion	4.24 (17,13)	"	64 (Indiv)	3.63-4.85 /14,67-19,59			
	Founding	6.27 (25,33)	weighted	13 (Clades)	5.73-6.81 / 23,15-27,51			
	Expansion	4.24 (17,13)	"	13 (Clades)	3.80-4.68 / 15,35-18,91			
	Coalescent	7.77 (31.39)	Max (12 nt)	12(Branches)	6.15-9.39 / 24,85-37,93			

X2a	Foundi ng	7.83 (31,63)	Normal	41 (Indiv)	7.11-8.55 / 28,72-34,54			
	Expansi on	3.83 (15,47)	"	40 (indiv)	3.11-4.55 / 12,56-18,38			
	Foundi ng	6.83 (27,59)	Weight ed	2 (Clades)	5.37-8.29 /21,69-33,49			
	Expansi on	2.83 (11,43)	"	2 (Clades)	1.37-4.29 / 5,53-17,33			
	Coalesc ent	11.00 (44,44)	Max (15 nt)	2(Branch es)	-39.82-61.82/-160,87-249,75			

The founding ages for the mtDNA Native American haplogroups, exposed above, coincide with the approximately 30 kya proposed in the first mtDNA studies (Bonatto and Salzano 1997; Torroni et al. 1994; Starikovskaya et al. 1998), and with the calibrated ages calculated by (Rieux et al. 2014), based on ancient mitogenomes. However, recent extant and ancient mitogenome studies (Bisso-Machado and Fagundes 2021; Llamas et al. 2016) and whole

genome-based studies (Moreno-Mayar et al. 2018; Willerslev and Meltzer 2021) strongly support post-LGM ages for the settlement of the Americas, although this is in part inspired by the most conservative archaeological ages proposed for the American settlement.

The age for the first population expansions calculated here, 17.86 kya, after the rigors of the LGM had lessened on the continent, does not differ much from those proposed by most authors. The difference is that for most authors, this expansion coincides with its arrival in America proper, while here, the arrival in America occurred much earlier, around 30 kya, before the glacial period had its worst effects.

Concerning the NRY, it deserves mention that, assuming a first colonization of the Americas about 15 kya, (Poznik et al. 2013) estimated a substitution rate for the Y-chromosome of 8.2×10^{-10} (95% CI: 7.2×10^{-10} – 9.2×10^{-10}) per site, year. However, doubling the time to 30 kya, as proposed here based on mtDNA, the substitution rate would be 4.1×10^{-10} per site, year which is very similar to that obtained by (Francalacci et al. 2013) of 5.3×10^{-10} (95% CI: 4.2 $X10^{-10}$- 7.0×10^{-10}) per site, year and both estimates falling within the limits of the mean 4.7×10^{-10} (95% CI: $2.1 X 10^{-10}$ – $9.5 X 10^{-10}$) per site, year proposed by myself based on past population sizes obtained under phylogenetic considerations (Cabrera 2024). As before, these results contrast with the

majority of the NRY younger coalescence ages estimated by other authors (The Genographic Consortium et al. 2016; Pinotti et al. 2019; Colombo et al. 2022; Paz Sepúlveda et al. 2022).

As YFull Tree v13.03.00 (YTree) has the most actualized NRY phylogeny and the most extensive phylogeography, it is increasingly used as a source of data in scientific articles, as in the present work. The YFull substitution rate (Adamov et al. 2015) is identical to the one calculated by (Poznik et al. 2013) under the premise that the human American settlement occurred around 15 kya, because of this, I have halved their substitution rate values when I used them. Under these requirements, NRY haplogroup C clades would arrive in the Americas in separate waves, C-MPB373 first at about 32.2 kya (95% CI: 28.8 – 35.6 kya), and C-P39/Z30536 second at about 26.2 kya (95% CI: 22.8 – 29.8 kya). The three main NRY haplogroup Q clades have very similar ages around 30 kya: Q-F746, 31.8 kya (95% CI: 28.4 – 35.2); Q-Z780, 31.0 kya (95% CI: 29.0 – 33.0); Q-M3, 29.8 (27.0 – 32.8). The age of arrival to the Americas of the rare clades Q-M1150 (30.0 kya (95% CI:26.6 – 33.4 kya) and Q-M378, 21.4 kya (95% CI: 17.6 – 25.4 kya), that include European and Asian lineages, is only tentative (https://www.yfull.com/tree/Q).

Regional heterogeneity for founding and expansion events

In a first approach, I made comparisons of the native haplogroups' founding and expansion ages between the North and South American subcontinents (Table 3). Except for haplogroup C1, the results reflect significantly older ages in South America, both in founding and expansion events.

Table 3. Differences in Founding(F) and expansion(E) ages between the North America and South America subcontinents

Haplogroup	t-value	df	p-value	oldest region
A2 - F	3.0397	1119	0.0024	South America
A2 -E	4.8326	1105	<0.0001	South America
B2 - F	1.0604	1099	0.2892	ns
B2 - E	3.1395	1083	0.0017	South America
C1 - F	1.4777	499	0.1401	ns

C1- E	0.4722	482	0.6370	ns						
D1 - F	5.7700	498	<0.0001	South America						
D1 - E	2.2473	466	0.0251	South America						
D4h3a - F	3.0245	69	0.0035	South America						
D4h3a - E	1.0115	59	0.3159	ns						

More detailed comparison between geographic regions, within and among subcontinents, using mean molecular age comparisons, pairwise genetic distances and Pearson correlation tests are described in supplementary tables (Supplemental Table S1.1 to S1.7 for A2; Supplemental Table S2.1 to S2.7 for B2; Supplemental Table S3.14, .7, .10, .13, .16 for C1b; Supplemental Table S3.2, .5, .8, .11, .14, .17 for C1c; Supplemental Table S.3.3, .6, .9, .12, .15, .18 for C1d; Supplemental Table S3.19 for C4c; Supplemental Table S4.1 to S4.6 for D1; Supplemental Table S4.7 to S4.9 for D4h3, and Supplemental Table S5.1 to S5.3 for X2a).

Haplogroup A2

Globally, A2 is the most abundant haplogroup, representing approximately 44% of the Native American population, but it has an uneven geographic distribution. The oldest founding ages for A2 (Supplemental_Table_S1.5) are found in Brazil and the Colombia-Isthmus area (≈ 34 kya), expanding first in Colombia around 23 kya. From the Colombian Isthmus region, significant latitudinal clines exist to Siberia (Slope -13.37; R^2 0.8396; P-value 0.0102), and to Tierra del Fuego (Slope 23.49; R^2 0.8091; P-value 0.0377). In addition, notice that in the most ancient samples of southern South America, mtDNA haplogroup A and B lineages were totally absent or very rare (Lalueza 1997; Moraga et al. 2000; Nakatsuka et al. 2020; Roca-Rada et al. 2021; Postillone et al. 2020) while they were detected in the most ancient samples recovered in northwestern North America more than 14 kya (Gilbert et al. 2008), and also were prominent in ancient samples from the Isthmus-Colombian area (Capodiferro et al. 2021). Another geographic peculiarity of haplogroup A2 is its significant abundance in the eastern areas compared to the western ones (Kemp et al. 2007; Raff et al. 2011; Rothhammer et al. 2017; Simão et al. 2021). It is also noteworthy that the oldest A2 haplotypes are found in North America and Brazil (Table S1.4). Furthermore, a secondary southward migration of some North American sub-lineages is also detectable. One of these lineages is

A2f, which is significantly older in North America (≈35,000 ya) than in Mexico (≈23,000 ya) (t=3.6172; df 22; p-value 0.0015).

For haplogroup A2, the shortest molecular genetic distances between regions (Table S1.3) were obtained for the pairs CSAm–SSAm (0.657), NWSAm–NESAm (0.682), and NCAm–NNAm (0.681). When these distances are represented in a PCoA (Fig. 1), it shows that the first principal coordinate places NESAm in a central position, leaving respectively at its left and right the remaining regions of North- and South-America. The second principal coordinate separates the regions of each sub-continent as latitudinal clines, mimicking the Pearson correlation results.

Haplogroup B2

With a global value of 24.0 %, hg B2 is the second-largest Native American haplogroup. The oldest founding (≈ 30 kya) and expansion (≈ 21 kya) regional values for this haplogroup (Supplementary_Table_ S2.6) are found in SSAm and CSAm. Going up from these southern areas, values descend in a clinal trend following a NWSAm orientation to reach Central and North America. This negative correlation between B2 molecular ages and latitude (Supplementary_Table_ S2.5) is highly significant (Slope: -13.57; R^2 0.5455; P-

values 0.0061). Unlike A2, Hg B2 is currently absent or very rare in northern North America, mimicking its practical absence in northeastern Siberia (Starikovskaya et al. 1998). Also, unlike A2 and D1, Hg B2 is more frequent in western continental regions than in the eastern ones (Bisso-Machado et al. 2012; Brandini et al. 2018). The oldest B2 haplotypes were found in the Andean region (Supplementary_Table_ S2.4).

The shortest genetic molecular distances were between SSAm-CSAm (0.492) and SSAm-NWSAm (0.500) pairs (Supplementary_Table_ S2.3). A PCoA bidimensional graphic representation of these distances shows NWSAm having a central position, with SSAm and CSAm at its left and NESAm and all northern American regions at its right, following a latitudinal cline as observed with the Pearson correlation (Fig. 2).

Haplogroup C1

Although in the beginning Hg C1 was considered as a homogenous clade, its three main native branches reflect different phylogeographic movements that need particular attention.

For Hg C1b, the oldest founding/expansion regional ages (Supplementary_Table_ S3.13) are in NESAm (≈ 29 kya/17 kya), with younger

ages going clinally down to Tierra del Fuego and up to Canada. The latter showing a significant trend (Slope -12.20; RSquare 0.8876; P-value 0.0166). These phylogeographic characteristics are rather similar to those found for HgA2. However, the longitudinal differentiation observed for the A2 clade is not seen in C1b. The oldest haplotypes for C1b are found in northern South America, including both Ecuador and Colombia, and reaching Mexico (Supplementary_Table_ S3.10). Furthermore, the shortest genetic distance between regions (Supplementary_Table_ S3.7) is that of the pair NWSAm-NESAm (0.522), followed by NWSAm-SSAm (0.605) and SSAm-CSAm (0.654). In this case, the PCoA graphic representation highlights the close relationship between NWSAm-Mexico and USA-Canada, leaving NESAm as the center from which southward movements across Brazil and northward to Central America occurred (Fig. 3).

For Hg C1c, the oldest founding/expansion regional ages (Supplementary_Table_ S3.14) have their center in NWSAm (≈ 34 kya / 26 kya). From this center, populations with younger ages conform southern and northern geographic transects ending in SSAm and Canada, respectively, the latter showing a significant correlation with latitude both for founding (Slope -14.79; R^2 0.8055; P-value 0.0152) and expansion ages (Slope -11.71; R^2 0.9629;

P-value 0.0005). These results partially resemble those obtained for Hg B2 in its North American subcontinent range.

Congruent with its Andean center, the oldest haplotypes for C1c are found in NWSAm and Mexico (Supplementary_Table_ S3.11).

The shortest genetic distances within Hg C1c (Supplementary_Table_S3.8) point to the existence of three geographic centers, the first represented by the CSAm-CAm pair (0.571), the second by the SSAm-NWSam pair (0.630), and the third by the NWSAm-Mexico pair (0.644). This pattern could be explained supposing NWSAm as the main center that spread downward to Argentina and Chile and upward to Mexico, and a later center that, from Brazil, reached Central America, deleting in this region the signal of the previous route that from NWSAm had reached Mexico. In this respect, it is worth mentioning that the Y-chromosome Q-M925, mainly Mesoamerican, has close phylogenetic ties with the Brazilian Q-Y26547 subclade (Colombo et al. 2022). This complex pattern is partially reflected in the PCoA plot (Fig. 4), which shows a latitudinal cline from NWSA to Canada passing through NESAm, with the anomalous inclusion of SSAm in it, and an independent close relationship between Central America and Brazil.

Hg C1d, with the oldest founding/expansion ages (Supplemental_Table_ S3.15) in CSAm and SSAm (≈ 38 kya / 33 kya), is the oldest C1 branch. In fact, it is the oldest Native American clade. Although without reaching significance, C1d shows a geographic descendant trend in population ages from North America to Central America (Supplemental_Table_S3.15). This pattern resembles the one found for Hg A2. In addition, it presents a significant latitudinal correlation from SSAm to Central America with the oldest founding (Slope -16.65; R^2 0.7649; P-value 0.0415) and expansion ages (Slope -13.29; R^2 0.6985; P-value 0.0382) in southern South America. This geographic trend in the South American subcontinent parallels the one described for Hg B2. The oldest haplotypes for C1d are found in CSAm and SSAm (Supplemental_Table_S3.12), reinforcing the existence of an ancestral southern South American center of expansion for this clade.

For C1d, the closest genetic distances (Supplemental_Table_S3.9) are between the SSAm-CSAm pair (0.591) and the NWSAm-NESAm pair (0.592). The latter one resembles the close genetic relationship between these regions, also observed for the C1b clade. The PCoA bidimensional representation of these distances (Fig. 5) shows a central group joining SSAm and CSAm with Central America, and two distant groups, one at the left joining NWSAm and NESAm, and the other at the right, grouping the northern North American populations

and Mexico. The independent and close genetic relationship between the South American regions and Central America is similar to the one found for C1c, which was explained supposing a later expansion from Brazil to Central America, while the close genetic affinity between NWSAm-NESAm is a replica of what was found for C1b.

Haplogroup C4c

Founding and expansion ages for C4c are around 25 kya and 17 kya, respectively (Table 2), but ages for its main branches are very different (Supplemental_Table_S3.19). The most developed branch, C4c1, has founding and expansion ages of approximately 16 kya and 12 kya, respectively, both after the LGM. However, the less frequent C4c2 sister branch has a founding age of 31 kya, more in line with the rest of the main Native American haplogroups, but a very recent expansion age of only about 3000 ya. C4c is a minoritarian haplogroup with a limited geographic range that, except for a sporadic occurrence in Colombia and in an ancient sample from Brazil, Loca Do Suin_9100 years old (Ferraz et al. 2023), is restricted to NNAm (Supplemental_Fig_S3). Thus, the anomalous results obtained for this lineage may be due to poor sampling and/or strong drift effects.

Haplogroup D1

For D1, the oldest founding ages (≈ 31 kya) are found in SSAm and CSAm (Supplementary_Table_S4.5). The earliest expansion ages are also found in the same regions but at different times (CSAm ≈ 23 kya; SSAm ≈ 18 kya). For D1 regional founding ages, a significant latitudinal correlation exists from SSAm to Central America (Slope -16.65; R^2 0.5431; P-value 0.0370), and a non-significant decreasing trend is observed from Canada to Central America (Supplementary_Table_S4.5). The oldest D1 haplotypes are mainly in NWSAm and then in SSAm (Supplementary_Table_S4.4).

The D1 shortest genetic distances are for the pairs SSAm-CSAm (0.618), USA-Canada (0.667), and those involving NWSAm with SSAm (0.768) and with NESAm (0.759). A PCoA plot (Fig. 6) graphically reflects these genetic relationships, grouping in a central position all the Mesoamerican and northern South American populations, well separated from two, in turn, very distant groups, composed of northern North American and southern South American populations, respectively.

The D1 phylogeographic data described above show a great similarity with the patterns obtained for haplogroups B2 and C1d.

Haplogroup D4h3a

For D4h3a, the oldest founding (≈ 30 kya) and expansion ages (≈ 22 kya) occurred in CSAm (Supplementary_Table_S4.8). Age comparisons between regions only reach significance when the oldest expansion ages of SSAm and CSAm are compared to those of NNAm and NWSAm (Supplementary Table S4.9). Living apart, one isolates from NESAm, the oldest D4h3a haplotypes are in NWSAm, Mexico, and consistently, in Chile (Supplementary_Table_S4.7). It has been proposed that D4h3a is a signal of a Pacific southward expansion (Perego et al. 2009). However, these results do not support that suggestion. It seems that D4h3a was more common in the past and most frequent in eastern areas than it is today.

Haplogroup X2

Haplogroup X2a is considered a minor Native American haplogroup with a limited spatial range confined to northern North America (Perego et al. 2009). The founding and expansion ages for haplogroup X2a are approximately 29 kya and 16 kya, respectively (Supplementary_Table_S5.1), and the oldest haplotypes are found in the X2a1 branch in samples from the North American Great Plains. Ages are significantly older in North America than in Canada (Supplementary Table S5.3). However, the detection of a divergent X2 haplotype in Canada (Perego et al. 2009), that defines a possible new Native American haplogroup, named X2g (Supplemental_Fig_S5), deserves mention.

Although the modern and ancient geographic distribution of X2a is biased to the northeast of North America (Perego et al. 2009), with sporadic incidences in Mexico (Peñaloza-Espinosa et al. 2007; Bodner et al. 2021), its oldest sample, the Kennewick Man, about 9000 years old, harboring a basal X2a haplotype, was exhumed in the northwestern Washington state (Rasmussen et al. 2015), raising the possibility that the X2a clade could be originated in the northern Pacific area (Perego et al. 2009).

NRY haplogroups

At a global scale, two major parental lineages with Pan-American distribution have been defined (Q-CTS1780/Z780 and Q-M3), both within haplogroup Q, the first in lower frequencies everywhere compared to the second. In addition, two minor lineages, belonging to haplogroup C, with a subcontinental range (C-MPB373 and C-P39/Z30536) have also been considered Native American founder lineages. The southern C-MPB373 has been reported in extant Colombians and Equatorians consistently (Karafet et al. 1999; Roewer et al. 2013; Mezzavilla et al. 2015; Villaescusa et al. 2021). The northern C-P39/Z30536 has been found in Canada and northern North America, reaching also Mexico (Perez-Benedico et al. 2016), and drawing a northern to southern gradient of decreasing diversity (Dulik et al. 2012). Although phylogenetically Q-CTS1780/Z780 is the oldest clade, the demographic expansion observed

after the LGM occurred, in males, almost exclusively within the Q-M3 lineage that simultaneously branched, mainly in Mesoamerica (Q-M925) and in South America (Q-M848), producing well differentiated regional subclades (Bortolini et al. 2003; Battaglia et al. 2013; Roewer et al. 2013; Geppert et al. 2015; The Genographic Consortium et al. 2016; Grugni et al. 2019; Pinotti et al. 2019; Gómez et al. 2021; Villaescusa et al. 2021; Colombo et al. 2022; Kemp et al. 2007; Paz Sepúlveda et al. 2022). Some of these minor Q subclades also deserve mention due to their geographic distributions and STR diversity. One of them is Q-MEH2, this lineage was detected in northeast Siberian Koryaks and its very close relationship to the extinct Paleo-Eskimo Saqqaq (Rasmussen et al. 2010) suggested the possibility that it could trace a recent migration from Siberia to America across the Bering Strait (Malyarchuk et al. 2011). However, Q-MEH2 has been recently detected in Nahua from southern Mexico, and from its STR variability, it was proposed that it arrived much earlier in Mexico than in northern America (Gómez et al. 2021). Another striking example is the Mexican Q-PV3 lineage, in principle, it was considered a minor branch of Q-L54, but its quite divergent haplotypic variability suggested the possibility that it might represent another founding Native American lineage (Gómez et al. 2021). This subcontinental differentiation of male ancestries is in support of diverse demographic histories in North and South America (Bortolini et al. 2003).

However, before to reach to any conclusion, because post-Columbian admixture heavily affected the Native American male genetic pool, in both North (Malhi et al. 2007) and South America (Resque et al. 2016), a consultation on what NRY ancient DNA analyses say is necessary. The picture was similar. Although Q-CTS1780 could be more abundant in the past in both subcontinents (Posth et al. 2018; Grugni et al. 2019; Ferraz et al. 2023), Q-M3 was also the predominant NRY lineage in all ancient DNA data (Bisso-Machado and Fagundes 2021). However, the detection in the past of today's extinct lineages (Posth et al. 2018; Colombo et al. 2022) supports the view that strong founding and drift effects occurred in NRY Native American lineages. These data do not fit well with the hypothesis of a rapid and recent migratory wave from the North to explain the prehistoric colonization of South America.

DISCUSSION

The early entry to the Americas of the founder Native uniparental lineages, more than 30 kya, proposed here, makes the hypotheses of a prolonged (Hoffecker et al. 1993; Tamm et al. 2007) or short (Pinotti et al. 2019) standstill in Beringia, a previous expansion of its ancestors into Asia (Wei et al. 2018; Sun et al. 2021; Ning et al. 2020), or the existence of a Pacific Coastal Route around 20 kya (Scheib et al. 2018; Davis and Madsen 2020; Li et al. 2023), unnecessaries.

The archaeological evidence for the presence of humans in the Siberian Arctic around 30 kya (Pitulko et al. 2004) and, possibly, since 45 kya (Pitulko et al. 2017) is in support of a possible early entrance in America of its Native ancestors. More difficult is to obtain a general acceptance, from archaeologists, of human occupation in the American continent before and around the LGM, but it seems very probable that this occurred around 16-18 kya in Monte Verde, Chile (Dillehay et al. 2015), in Santa Elina site, Central Brazil during the LGM, at Chiquihuite Cave in Central Mexico about 30 kya (Ardelean et al. 2020), in the Colorado Plateau more than 36 kya (Rowe et al. 2022), in White Sands, New Mexico, during a stratigraphic record that spans from 24 kya to 17 kya (Holliday et al. 2025), and in Bluefish Caves, Canada, approximately 24 kya (Bourgeon et al. 2017).

Ancient genome-based studies have described the ancestral Native American population as an admixture of northern Eurasians and northern East Asian populations (Raghavan et al. 2014). The same dual contribution was proposed previously using NRY information (Karafet et al. 1999; Lell et al. 2002; Roewer et al. 2013), and an identical conclusion has been reached here based on mtDNA data. Genomic research proposed that the ancestral Native Americans arrived on the New Continent as an already admixed population (Moreno-Mayar et al. 2018). However, although coalescence ages do not show

significant differences between haplogroups, the NRY phylogeny is more compatible with the arrival of two consecutive waves, the first carrying C-MPB373 and Q-Z780 lineages, and the second being the carriers of C-P39/Z30536 and Q-M3 lineages. The evidence is weaker for mtDNA, but, as mentioned before, the fact that the basal branches joining haplogroups C and D to their respective Asian nodes are significantly shorter than those of A and B could be taken as an argument to favor a first arrival into America of the former pair. Another clue could lie in the Southern Cone of South America, the region furthest from the entrance to the continent. Ancient and current sampling of the region discovered that haplogroups C and D were the first and unique lineages reaching the area being even today the most prevalent (Lalueza 1997; García-Bour et al. 2000; Moraga et al. 2000; Demarchi et al. 2001; Nakatsuka et al. 2020; Postillone et al. 2020). The prevalence of haplogroups C and D has also been observed in Amazonian populations from Brazil (Merriwether and Ferrell 1996), which could be the origin of an early southward migration. In addition, it has been demonstrated that mtDNA diversity increases from north to south for haplogroups C1 and D1, whereas North American populations show higher diversity levels for haplogroups A2 and B2 (Lee and Merriwether 2015). Moreover, in North America, haplogroups A, B, and X have undergone population clinal expansions, while there is no

evidence of this spread in haplogroups C and D (Malhi et al. 2001). These patterns are all compatible with two consecutive waves of migration. However, these results could also be attributed to drift effects, as samples in other regions also show the total absence of some founder mtDNA haplogroups, as is the case of the Kuna from Panama in which the absence of C and D has been documented (Batista et al. 1995) and, contrarily, the Ayoreo from Bolivia showed a total absence of A and B (Dornelles et al. 2004). Based on the distribution of two rare mtDNA haplogroups, D4h3 and X2a, the first spreading into the Americas along the Pacific coast and the second entering through the ice-free corridor, two migratory waves from Beringia were proposed (Perego et al. 2009). However, for the case of D4h3, the authors admitted that the fact that it is very rare in North America and more frequent and with greater variation in South America, needs some additional explanation (Perego et al. 2009).

Another proposition, based on genome studies, is that after it arrives in

North America, the native ancestral population splits into two distinct branches known as South Native Americans (SNA) and North Native Americans (NNA) (Moreno-Mayar et al. 2018) or their approximate counterparts, Ancestry A (ANC-A) and Ancestry B (ANC-B), respectively (Scheib et al. 2018). While the northern ancestry is geographically rather limited to Canada and eastern North

American regions, the southern ancestry is detectable in the oldest genomic sequence sampled, Anzick1_12700 years old from Montana (Rasmussen et al. 2014), and in the Spirit Cave sample from Nevada, 10700 years old (Moreno-Mayar et al. 2018) extending southwards to Mesoamerica and southern America (Moreno-Mayar et al. 2018). There are mono-parental markers that share the geographic range of the NNA genomic ancestry as the mtDNA haplogroups X2a (Brown et al. 1998) and C4c (Kashani et al. 2012) and the NRY lineages C-P39 (Pinotti et al. 2019), Q-Y4276 (Grugni et al. 2019). The situation is much more complex when the transcontinental distribution of the southern component of the Native American genome has to be explained. The initial dual explanation of southward Pacific coastal expansion in which the successive populations could be represented as admixtures, in different proportions, of the SNA and NNA components (Scheib et al. 2018) was proved to be insufficient (Posth et al. 2018). First of all, the uniqueness of the northernmost SNA component, represented by the Clovis associated Anzick1 sample (SNA1), could not explain the genomic compositions of many south American populations and an additional, new and dominant non-Clovis SNA2, represented in part by the Spirit Cave ancestry, had to be added to properly explain the genome composition of many southern populations. Entering Mesoamerica, the Mexican Mixe needed the addition of gene flow from an

unsampled population to be fully represented (Moreno-Mayar et al. 2018). Moreover, several yet undiscovered genomic ancestries had to be added to satisfactorily model the genomic structure of many South American well-differentiated regional populations. An enigmatic case was the presence in Amazonian Surui, Karitiana, and Xavante tribes of a divergent ancestry related to Australasian people (Raghavan et al. 2015; Skoglund et al. 2015). This component, named "population Y," was confirmed in ancient Brazilian samples as Sumidouro5, approximately 10000 years old (Moreno-Mayar et al. 2018). At least one mtDNA haplotype had to accompany the Australasian ancestry, and, due to its southernmost distribution, a lineage from haplogroup B seems to be the best candidate. However, with which ancestral Asian population it entered the New World is difficult to determine since, on the one hand, basic lineages of B were present in eastern Asia around 40 kya, as attested by the Chinese Tianyuan sample mtDNA analysis (Fu et al. 2013). Moreover, the Australasian ancestry detected in the South American Amazonians is also shared by the Tianyuan sample (Yang et al. 2017). Therefore, the Australasian component could enter America with the descendants of a population deeply settled in eastern Asia, which had ancient Australasian ties, but, likewise, it could enter with the carriers of mtDNA haplogroup B from central Eurasia. The Isthmus-Colombian area was also a center of genome complexity, and a Pleistocene

ancestry was brought to the Panamanians, and it remained restricted to the Isthmus area (Capodiferro et al. 2021), and hunter-gatherers from the Colombian Altiplano also harbored an unknown basal lineage that seems derived from the initial South American genomic radiation (Krettek et al. 2025). In the northwest side of South America, a previous undetected ancestry was characteristic of northern Ecuador and Colombian populations (Barbieri et al. 2019), and Native Peruvians from the Lake Titicaca region showed a Siberian component also present in ancient North Americans as Anzick1, Kennewick, or Saqqaq (Lindo et al. 2018). The complex ancestries found in South America forced some followers of the rapid post-glacial colonization of America to make some cautionary comments, as that a possible northward radiation from South America to explain the Pacific migratory wave would require a more complex history (Posth et al. 2018), or that the SNA dispersal pattern south of the Continent's ice sheets involved complex admixture events between earlier established populations (Moreno-Mayar et al. 2018). The analysis of the uniparental markers realized in this study offers an alternative hypothesis that could integrate all the genomic information under a different perspective.

If we accept that the entry of Asian populations that gave rise to Native Americans occurred before 30 kya, the history of human settlement in America can be told differently. It is known that since its arrival, the weather gradually

worsened, covering with ice the regions near the Arctic and Antarctic (Clark et al. 2009), clearing the Amazon and Central American rainforests (Häggi et al. 2017), and transforming most of the territory into forest and dry steppes (University of Geneva, Switzerland et al. 2001). During this harsh phase, humans had to live in small isolated bands, and adapt to different glacial refugia that would ensure their survival, therefore, leaving few archaeological traces of their existence. When the weather improved, these isolated bands grew demographically and spread to new, now habitable territories. Thus, differently from the fast southern wave colonization, the American continent was not void of previous human occupation, and the post-LGM colonization of the New World was not accomplished by one or several consecutive spreads from northern North America to settle first Mesoamerica and after South America, but from more or less contemporaneous radiations in multiple geographic centers the majority of which, uniparental markers, situate in South America. One of the main expansion centers for mtDNA is the northwest Colombian-Isthmus area, where double latitudinal younger clines northwards and southwards are detectable for haplogroups A2 and C1b. A similar trend in A2 relative frequencies has also been detected in the same area (Keyeux et al. 2002). Another apparent center is located in the Andean region where mtDNA haplogroup C1c shows its oldest foundation and expansion ages, with younger

geographic clines running up to Canada and down to SSAm. This pattern parallels the one found for haplogroup B that expanded from Chile and the Andean region, entering into Mesoamerica. However, it seems that, at least in part, the B2 cline is the result of its displacement in North America by other mtDNA lineages since it was present very early in northeast Asia as an ancestral B lineage (Fu et al. 2013) and in Alaska as a basal B2 lineage (Tackney et al. 2015) to practically disappear later in both areas. The highest frequencies and genetic diversity values for haplogroup B2 have been found in the Andean Altiplano (Gayà-Vidal et al. 2011; Taboada-Echalar et al. 2013), in the Bolivian Piedmont (Corella et al. 2007), and in the Argentinian Gran Chaco (Demarchi et al. 2001). Although the classic route to explain the B2 cline is to assume a descent from California (Taboada-Echalar et al. 2013), South American origins, as proposed here, have also been previously formulated from the Peruvian Andes (Arencibia et al. 2025) or the Chilean Southern Cone (Bravi et al. 2024). Also, as B2, C1d is another mtDNA clade that shows its most ancient founding and expansion ages in the southern Cone, going up, with younger values, to Central America, presenting a significant geographic correlation. In North America, some lineages of this clade present an expansion age descendant trend that ends in Central America; likewise, the observed trend for haplogroup A2 sub-lineages. C1a (Tamm et al. 2007) is a northeast Asiatic

branch of C1 that, together with the minor European sub-clades C1e (Ebenesersdóttir et al. 2011) and C1f (Der Sarkissian et al. 2014), suggests a subsequent retro-migration from America (Tamm et al. 2007). Applying the evolutionary rate proposed here, the founding age in Asia of C1a is 26.02 kya (95% CI: 18.58-33.49 kya), which would signal a return to the Asian continent with worsening weather conditions. Its expansion, in the Asian continent, around 13.9 kya (95% CI: 6.46-21.37 kya) would indicate a Mesolithic spread like the one found for the northeast European C1f clade (Der Sarkissian et al. 2014). This possible early American reflux can also be deduced from the phylogeny and phylogeography of the Y-chromosome haplogroups C-L1373 (Pinotti et al. 2019; Colombo et al. 2022) and Q-L54 (Grugni et al. 2019; Pinotti et al. 2019).

Another mtDNA haplogroup that signals to the southern Cone region as an important center of expansion is D1, showing a significant negative correlation from this area to Central America, with mean coalescent ages younger going upwards, although with a longitudinal bias leaning towards central and eastern South American regions. D1 has the greatest frequencies at the eastern areas of the Andean region, with regional ancient clades in Central Argentina (García et al. 2012) and the southern Cone (Bodner et al. 2012). Likewise, for A2 and C1d, a southward expansion from Canada to Central America is detected for

D1. These expansive movements could be the cause of the B2 retraction observed in North America. The suggestion that mtDNA haplogroup D4h3a would confirm the hypothesis of a Pacific coastal route for the peopling of America (Perego et al. 2009), subsequently reinforced by genomic studies (Moreno-Mayar et al. 2018), is not supported by the evidence presented here. The oldest founding and expansion ages for D4h3a are found in SSAm and CSAm, being significantly older than those observed in NWSAm and NNAm (Supplementary Table S4.9). Furthermore, although today absent, D4h3a has been detected in ancient samples from Alaska, Shuká-Káa, 10.3 kya (Lindo et al. 2017), British Columbia, Canada, 939, 6.5 kya (Lindo et al. 2017), and Montana, Anzick 1, 12.6 kya (Rasmussen et al. 2014). Furthermore, it was observed in ancient remains from the Brazilian Amazon, Lagoa Santa, 10 kya (Moreno-Mayar et al. 2018). This pattern is very similar to the one observed for B2. Thus, better than modelling a southward Pacific Coastal expansion, both markers seem to represent the remnants of a more extensive occupation in the past, which was displaced by more recent human migrations.

A particular commentary deserves the Amazonian area (CSAm). It participated in all mtDNA centers of radiation commented above, and it is described as a large reservoir of mtDNA deep lineages (De Souza et al. 2025; Di Corcia et al. 2017), and stands out for the ancient presence of very basal Y-chromosome

lineages as C-L1373*(Colombo et al. 2022), or the detection there of lineages that were considered exclusive to the northern subcontinent as Q-Y4303*(Grugni et al. 2019). Moreover, this genetic complexity has been corroborated by whole-genome-based studies in ancient specimens (Moreno-Mayar et al. 2018) and in extant populations (Ribeiro-Dos-Santos et al. 2020; Capodiferro et al. 2023). This genetic landscape has its correlate in the linguistic richness of the area. It is enough to point out that of the 71 linguistic isolates described for the double continent, more than 45% have been found in Brazil compared to 15% observed in North and Mesoamerica as a whole (Blench and Cb). It is expected that future archaeological discoveries in the area will unequivocally confirm human settlements much older than those currently accepted. Again, the differences found in genetic and linguistic complexity between the northern (NNA) and the southern (SNA) American branches make the simultaneous colonization of both subcontinents from a North American southward spread very improbable.

MATERIAL AND METHODS:

Material

For the mtDNA phylogenetic and phylogeographic analyses, I searched partial and complete mitogenomes at the NCBI GenBank, www.ncbi.nlm.nih.gov/genbank/; Mitomap,www.mitomap.org/MITOMAP; EMPOP, https://empop.online; Ian Logan 2023, www.ianlogan.co.uk/sequences_by_group/haplogroup_select.htm; Genome Warehouse (GWH) public repository, https://bigd.big.ac.cn/gwh, and AmtDB, www.http://amtdb.org; in addition, for phylogeographic analyses only, I also searched YFull, MTree. The NRY phylogenetic and phylogeographic analyses were based on the ISOGG database, http://www.isogg.org/tree/, YFull YTree v13.03.00, YTree, and the data compiled by other authors (Wei et al. 2018; Grugni et al. 2019; Pinotti et al. 2019; Gómez et al. 2021; Colombo et al. 2022; Paz Sepúlveda et al. 2022). For brevity, the Y-chromosome lineages in the text are referred to by the letter of their basic haplogroup and their terminal mutation.

Phylogeny

Maximum parsimony intraspecific phylogenetic trees (Bandelt et al. 1999) were constructed by hand, and haplogroup nomenclature was according to the PhyloTree database Building 17, http://www.phylotree.org. Haplogroup classification was confirmed using HaploGrep 3 3.2.1, https://haplogrep.i-med.ac.at. (Weissensteiner et al. 2016). New subgroups not classified in

PhyloTree were provisionally named by the first letter and digit of the primary haplogroup to which they belong, followed by an asterisk and consecutive numbers. When a more formal nomenclature exists in YFullMTree, both labels are shown on the corresponding tree.

Evolutionary rates

It is well known that the mtDNA evolutionary rate suffers a time-dependent effect, slowing down going backwards (Henn et al. 2008). The main cause of this effect has been attributed to differences in population size, with the smallest sizes in Paleolithic times and an exponential growth in present times (Cabrera 2021a). To compensate this anomaly, I have used an estimated mitogenome Paleolithic substitution rate of 1.5×10^{-8} (95% CI: 0.42 - 9.07 x 10-8) per site per year (assuming a mtDNA genomic length of 16,500 base pairs) that is, one substitution in every 4,040 years (Cabrera 2024), to calculate haplogroup coalescent ages for the initial prehistoric settlement of the Americas. Following a similar reasoning, a putative mean evolutionary rate of 0.47×10^{-9} (95% CI: 0.21 – 0.95 x 10-9) per site per year was proposed to calculate coalescent ages of the Y-chromosome (Cabrera 2024).

Ages

I have performed three approaches to calculate haplogroup coalescent ages: 1) using statistics rho (Forster et al. 1996) and Sigma (Saillard et al. 2000) on individuals within haplogroups, and the evolutionary rates proposed above; 2) using clades within the main haplogroups to calculate a pooled mean to use normal average and standard error statistics to calculate mutation mean and standard errors for haplogroups ; 3) As the sample sizes of the clades have large differences, I calculated weighted averages and weighted standard errors to use them with normal statistics as above; 4) One of the most commented genetic drift effects is the possibility that haplotypes in low frequency become much more frequent in a population. However, it should be noted that this possibility is an exception more than a rule; it is normal for rare haplotypes, located at the tails of a distribution, to become extinct after drift, whereas those around the mean to augment in frequency. As it is evident that the Native American founding haplogroups suffered genetic drift fluctuations in the past, I have used the most divergent lineages left within haplogroups as possible past means to calculate their coalescence ages (Cabrera 2022)(Cabrera 2021a).

Phylogeography

For phylogeographic purposes the American continent has been divided into the following seven geographic regions: 1) North northern America (NNAm)

including Alaska and Canada; 2) North America, comprising the entire United States (Nam); 3) Mesoamerica, including Mexico, Belize, Guatemala, El Salvador, Honduras and Nicaragua (Mam); 4) Northeastern south America or Isthmus-Colombian area, comprising Costa Rica, Panama and Colombia; 5) Northwestern South America (NWSAm) or Andean region, including Ecuador, Peru and Bolivia; 6) Central South America (CSAm) including Brazil, Paraguay and Uruguay; 7) South southern America (SSAm) joining Argentina and Brazil. In addition, within regions, when necessary, it has been distinguished between inland vs coastal Pacific and coastal Atlantic areas and between Highland Andes vs Amazonian areas.

To reinforce the phylogenetic and phylogeographic analyses, I have implemented some simple algorithms, which I describe below.

Genetic distances

Besides the molecular distance based on the number of mutations between two homologous DNA sequences (F_{ST}), I have implemented a phylogeographic distance (D_{XY}) based on the genetic affinity (I_{XY}) between regions X and Y, measured as the number of times they form sister branches within a haplogroup tree (n_{XY}) versus the number of times each one participates as a sister branch within the haplogroup being measured (n_X and n_Y):

Then D_{XY} is calculated as $1 - I_{XY}$.

In addition, when searching for sister branches between X and Y regions within a haplogroup, I also recorded the cases in which one sequence, belonging to one of the regions involved, was a direct outgroup of the pair, signaling it as ancestral with respect to the other region considered derived. The significance of this relation was checked by observed-expected chi-square statistics.

Also, for phylogeographic purposes, I have searched, within each major Native American founder haplogroup, the regions where their oldest founder sub-haplogroups are found and the regions from which they first expanded.

Finally, to find the Asian centers from which the ancestors of the major American founder clades might have departed, I have looked for the most ancient Asian sequences (mutationally less evolved) that come from the Asian-American shared phylogenetic nodes, and which are the regions where these sequences are found.

Other statistics

Analysis of molecular variance (AMOVA), correlation/linear regression, and graphical Principal coordinates analysis (PCoA) have been implemented with the GenAlEx 6.5 software (Peakall and Smouse 2012). Descriptive normal statistics, 95% confidence intervals of means, and two-tailed t-tests were

implemented with GraphPad https://www.graphpad.com/quickcalcs/. Weighted mean and standard errors were obtained with the Weighted calculator (https://calculatodo.com).

False positive correction

As I have performed multiple statistical tests for each founder haplogroup, I have applied a Bonferroni p-value adjustment. However, as the Bonferroni correction is very conservative, I have also used the Benjamin and Hochberg 1995(Benjamini and Hochberg 1995) false discovery rate (FDR) algorithm as a less restrictive alternative.

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