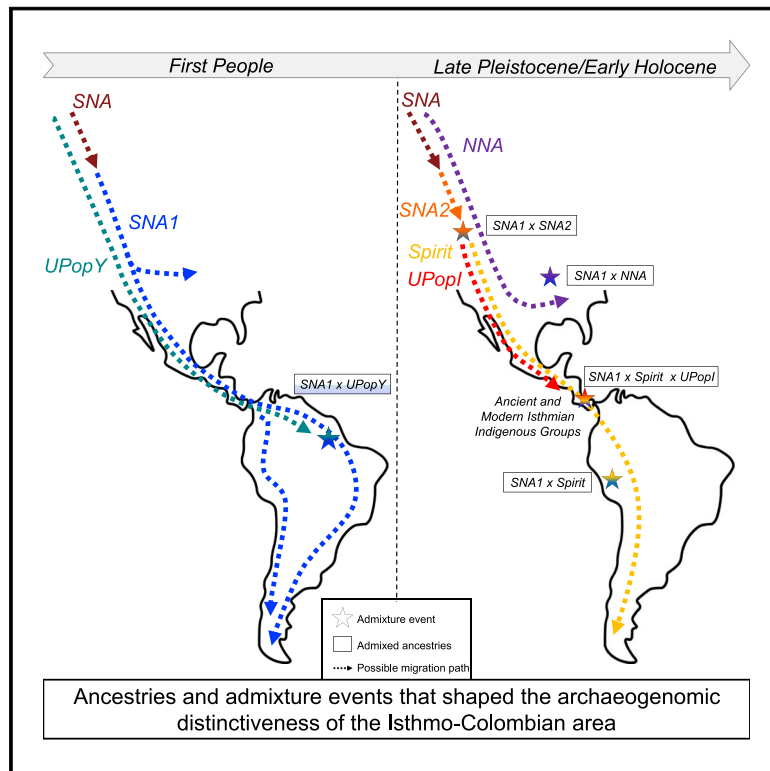


Archaeogenomic distinctiveness of the Isthmo-Colombian area

Graphical abstract



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In brief

Pre-contact and modern genomes from Panama highlight the distinctiveness of the Isthmo-Colombian area; detail number, source, and impact of Indigenous American genomic ancestries at the continental level; and explain complex pre-Hispanic burials.

Highlights

- Ancient Isthmian genomes address anthropological questions on pre-contact burials
- The comparison with modern Panamanians highlights genomic structure on the Isthmus
- A genomic component drives the Isthmian groups on a distinctive variability axis
- A previously unknown Pleistocene ancestry identified in the Isthmo-Colombian area



Article

Archaeogenomic distinctiveness of the Isthmo-Colombian area

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SUMMARY

The recently enriched genomic history of Indigenous groups in the Americas is still meager concerning continental Central America. Here, we report ten pre-Hispanic (plus two early colonial) genomes and 84 genome-wide profiles from seven groups presently living in Panama. Our analyses reveal that pre-Hispanic demographic events contributed to the extensive genetic structure currently seen in the area, which is also characterized by a distinctive Isthmo-Colombian Indigenous component. This component drives these populations on a specific variability axis and derives from the local admixture of different ancestries of northern North American origin(s). Two of these ancestries were differentially associated to Pleistocene Indigenous groups that also moved into South America, leaving heterogenous genetic footprints. An additional Pleistocene ancestry was brought by a still unsampled population of the Isthmus (UPopI) that remained restricted to the Isthmian area, expanded locally during the early Holocene, and left genomic traces up to the present day.

INTRODUCTION

Archaeological and genetic evidence suggests that the peopling of sub-Arctic America started from Beringia before, during, and

immediately after late Glacial times (Achilli et al., 2018; Ardelean et al., 2020; Becerra-Valdivia and Higham, 2020; Braje et al., 2017; Skoglund and Reich, 2016; Waters, 2019; Yu et al., 2020). Initial settlement attempts were followed by a more widespread



peopling that reached southern South America as early as ~15 thousand years ago (kya) (Dillehay et al., 2017). Recent studies of ancient and modern genomes describe a complex scenario prior to European contact with multiple migrations from Beringia, as initially suggested by mitochondrial DNA (mtDNA) data (Achilli et al., 2013; Brandini et al., 2018; Gómez-Carballa et al., 2018; Llamas et al., 2016; Perego et al., 2009; Perego et al., 2010; Tamm et al., 2007) as well as demographic spreads and admixture events along the two continents (Flegontov et al., 2019; Moreno-Mayar et al., 2018; Posth et al., 2018; Scheib et al., 2018; Schroeder et al., 2018). The great majority of ancestries in early Native Americans (NAs, here used to indicate Indigenous groups) derive from an ancestral Beringian population(s) that differentiated sometime between ~22 and ~18 kya and likely exhibited genetic sub-structure that may explain the initial late Glacial migration(s) as well as the spread of the so-called UPopA (unknown population in the Americas) whose legacy reappears in Central America ~8.7 kya, leaving signs in the gene pool of the Mixe (Moreno-Mayar et al., 2018). In unglaciated eastern Beringia/northern North America, the first peoples split into two branches called Northern NA (NNA, or ANC-B) and Southern NA (SNA, or ANC-A). The most ancient representatives of SNA are individuals who were living on both sides of the Rocky Mountains more than 10 kya: the Clovis-associated Anzick-1 and the Spirit Cave individuals associated with Western Stemmed technology. Ancient individuals carrying SNA ancestries crossed the Panama land bridge and entered South America. Their fast spread along the southern continent is evidenced by the earliest archaeological human presence in the Southern Cone at 14.6 kya and by ancient human genomes dating more than 9 kya on both sides of the continent: at Cuncacha (Peru) and Los Rieles (Chile) on the Pacific and Lapa do Santo and Lagoa Santa (Brazil) on the Atlantic. Another UPop (UPopY) with Australasian ancestry may have contributed to the early peopling of South America as recognized in one sample from the Lagoa Santa site and in some Amazonian groups that experienced isolation events (e.g., Surui and Karitiana) (Moreno-Mayar et al., 2018; Skoglund et al., 2015).

However, the demographic dynamics underlying many of these events, before and after European contact, are still uncharacterized, especially at the regional level (Fernandes et al., 2020; Lindo et al., 2017; Nakatsuka et al., 2020; Nägele et al., 2020). The Panamanian isthmus lies between the Atlantic and Pacific oceans and connects the two American continents. It was the only land bridge during the initial peopling of South America and has remained a crossroads of goods, technologies, ideas, and peoples throughout history, including more recent colonial times (Cooke, 2005; Cooke et al., 2019; Hernández Mora et al., 2021). In light of Panama's geographic location, the archaeogenomic study of its past can reveal its demographic history, including movements between North and South America.

Ethics and community engagement

This study involves international collaborative efforts that bring together archaeologists, geneticists, historians, anthropologists, and computer engineers to incorporate existing knowledge with genomic information about pre-Hispanic as well as modern Indigenous individuals from the Isthmus of Panama. It was possible with the support of local authorities and Indigenous

peoples of Panama and centrally involved local co-authors of the present paper (J.R., T.M., M.T., J.M.P., R.C., J.M., and J.G.M.) with years of experience in the Isthmo-Colombian region. Samples from the ancient individuals were collected for the ArtEmpire European Research Council (ERC) project (Consolidator Grant CoG-2014 no. 648535) in collaboration with the Patronato Panamá Viejo (PaPV) as established by the Convenio Específico de colaboración entre el Patronato Panamá Viejo de la República de Panamá y la Universidad Pablo de Olavide, de Sevilla, España, signed on January 20th, 2016. Excavations were undertaken with the permission of the Republic of Panama's Instituto Nacional de Cultura, Dirección Nacional de Patrimonio Histórico (DNPH), Resolución DNPH no. 139-16 of November 11th, 2016, and resolución DNPH no. 006-18 of January 8th, 2018. Selected samples from bone and teeth were exported to Pavia (Italy) and Mannheim (Germany) in accordance with the Permission of the Republic of Panama's Instituto Nacional de Cultura, Dirección Nacional de Patrimonio Histórico, Resolución no. 080-17 DNPH of April 19th, 2017, and resolución no. 304-18 DNPH of September 26th, 2018. Even though no ties to the pre-Hispanic inhabitants have been stated, we are engaging local Indigenous communities to present the information from this study and to seek their views. In general, the project has been designed to maximize opportunities for public engagement, as testified by ongoing meetings with local interest groups to discuss research design and findings. In order to increase positive social impact, some information on the ancient samples are publicly available in the ArtEmpire's database, translated into Spanish to increase accessibility (<https://artempire.cica.es/>) (Aram et al., 2020), and a documentary also has been released (<https://www.youtube.com/watch?v=5BmxxpS4oks>).

The collection of the modern Indigenous samples was approved by the Comité de Bioética de la investigación del Instituto Conmemorativo Gorgas and undertaken by the Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES, Gorgas Memorial Institute for Health Studies) of Panama. The ICGES explained the project to community leaders in their native languages and sent biological samples to the Department of Biology and Biotechnology of the University of Pavia for DNA extraction and analysis in agreement with the memorandum of understanding (written in English and Spanish) signed on August 9th, 2016. All experimental procedures and individual written informed consent forms were also reviewed and approved by the Ethics Committee for Clinical Experimentation of the University of Pavia, Board minutes of October 5th, 2010, and April 11th, 2013. We are particularly grateful to and acknowledge the ancient and modern people who shared their DNAs.

Panama: archaeology and history

Paleoecological and archaeological data point to a continuous human habitation of the Isthmo-Colombian area from approximately 16 kya (Cooke et al., 2013; Ranere and Cooke, 2020). Clear evidence for the cultivation of domesticated plants, including maize (*Zea mays*), manioc (*Manihot esculenta*), and squash (*Cucurbita moschata*), dates back to more than 8–4.5 kya (Linares, 1977a, 1977b; Linares and Ranere, 1980; Linares et al., 1975; Piperno, 2011; Ranere and Cooke, 2020), while Panama's first pottery (Monagrillo ware) appeared about 4.5 kya

(Martín et al., 2015, 2016). By 3 kya, the area's western region possesses all the characteristics of a coherent historical unit (Greater Chiriquí, which extended into present-day Costa Rica), while this consensus is not available for the central and eastern regions, often termed Greater Coclé and Greater Darién (see specific section in STAR Methods for further details).

From approximately 500 BCE to 1,500 CE, relations among neighbors oscillated between cooperation driven by exchange and trade and conflict over land and resources. Although polities often called chiefdoms expanded and contracted, there is no evidence for aggressive empire building as in Mexico or the Andes (Helms, 2014). On the eve of the Spanish invasion, historians and most archaeologists agree that much of the central and eastern Isthmus was inhabited by Indigenous polities that spoke languages in the Nuclear Chibchan family (with variants of languages in the Chocoan family probably also spoken on the Pacific side) and used the "language of Cueva" as a *lingua franca* (either a trade language or a group of vernaculars) in a linguistically complex region, much as the Huëtar did in Costa Rica (Cooke 2016; Costenla, 2012; Romoli, 1987). Based on partial and fragmentary data, historians have ventured estimates of the pre-Hispanic population in those areas where the language of Cueva was spoken at European contact from 130,000 to 240,000 people and archaeologists have identified specific villages capable of sustaining up to 2,400 inhabitants (Cooke, 2005; Cooke et al., 2019; Romoli, 1987). After nearly one millennium of less destructive war and trade among neighbors, European incursions provoked a rapid decline in the region's Indigenous populations. However, not all Indigenous groups experienced simultaneous demographic decline. Historical records suggest that an expansion among the Guna followed the reduction of other Indigenous groups (Castillero Calvo, 2017).

Panama: Genetics

To date uniparental systems have been examined to assess the genetic history of Panama: mtDNA data identified specific lineages predating the Clovis technological horizon (13.2 kya), while the comparison with Y chromosome data revealed a sex bias during the colonial period consistent with "more native men perishing or being deprived of reproductive rights than women" (Grugni et al., 2015; Perego et al., 2012). Similar to mtDNA data, patterns of regional genetic continuity in some Indigenous American (IA) communities have been inferred from the analysis of nuclear genomes from continental Central America (Reich et al., 2012), but without ancient DNA (aDNA) data from the Isthmian land bridge.

To refine the human genetic history of the Isthmus, for the first time we have directly tested and analyzed autosomal markers of both pre-Hispanic human remains and contemporary Indigenous groups from Panama. Twenty ancient individuals (13 of them pre-Hispanic and seven colonial) were sampled from seven different archaeological excavations along the Pacific coast of Panama City, located from the residential area of Coco del Mar to the remnants of Old Panama's Cathedral in Panamá Viejo, an area of pre-Hispanic inhabitation and the site of the colonial city from 1,519 to 1,671 CE (Hernández Mora et al., 2021). Modern sampling for a total of 84 individuals, 76 self-identified as associated with five different Indigenous groups plus four self-

designated "Moreno" and four self-identified "Mestizo" individuals (Figure 1A; Table S1), took place in Panama City as well as in the provinces and Indigenous territories.

RESULTS

Although the tropical environment and the proximity of the excavation sites to the ocean, with recurrent flooding, challenge the possibility of DNA preservation, we were able to obtain some of the first reliable aDNA data from the Isthmus. Starting from the initial collection of samples from 20 ancient individuals, we eventually assembled ancient low-coverage ($\geq 0.01X$) genomes from 12 unrelated individuals (one female and eleven males), including ten from pre-Hispanic times (radiocarbon dated from 603 to 1,430 CE). Molecular decay analyses demonstrated the poor preservation of endogenous DNA, but error rate and validation tests confirmed the reliability of the retrieved genomic data (Table S2). In order to characterize the genetics of Isthmian individuals with the greatest possible spatial range and temporal depth, the 12 ancient genomes were compared with genome-wide data from 74 unrelated modern Panamanians and to available modern and ancient data by assembling different datasets (STAR Methods; Table S3).

Uniparental lineages of pre-Hispanic Panamanians

The evaluation of uniparental markers revealed the presence of the "pan-American" mtDNA haplogroups A2 and B2 in the pre-Hispanic samples, while two haplogroups, H1j1a and L2a1c2a, typical of Europeans and sub-Saharan Africans, respectively, were identified in the samples taken from colonial ancient individuals (Figure S1A; Table S1).

The most represented mtDNA haplogroup of pre-Hispanic Panamanians, A2af1, was previously identified (as A2af) at high frequencies among present-day Panamanians, mainly in the Comarca of Guna Yala (Perego et al., 2012). It is characterized by the so-called "Huëtar deletion," a peculiar 6-bp control-region deletion initially detected in the Chibchan-speaking Huëtar from Costa Rica (Santos et al., 1994).

The eight pre-contact Y chromosomes are positive for the L54 marker, which characterizes all the Indigenous American branches of haplogroup Q (Figure S1B). Two individuals (PAPV118, PAPV175) were further sub-classified as Q1b1a1a-M3 and one (PAPV117) as Q1b1a1a1-M848, the most frequent haplogroups among Indigenous peoples of the Americas (Grugni et al., 2019; Pinotti et al., 2019).

Archaeological and anthropological significance of two burials in Panamá Viejo and Coco del Mar

An initial evaluation of the ancient low-coverage genomes made it possible to address long-standing anthropological and archaeological questions regarding the possible genetic relationships among individuals buried together. These cases included the ten human remains (one adult, nearly complete, female skeleton with nine adult male skulls beneath and around her) recovered from a pre-Hispanic burial denominated Tumba 1 underneath the Plaza Mayor of Panamá Viejo, and a similar burial at Coco del Mar (approximately 1 km to the west of Panamá Viejo), where a female skeleton was found accompanied by three male crania

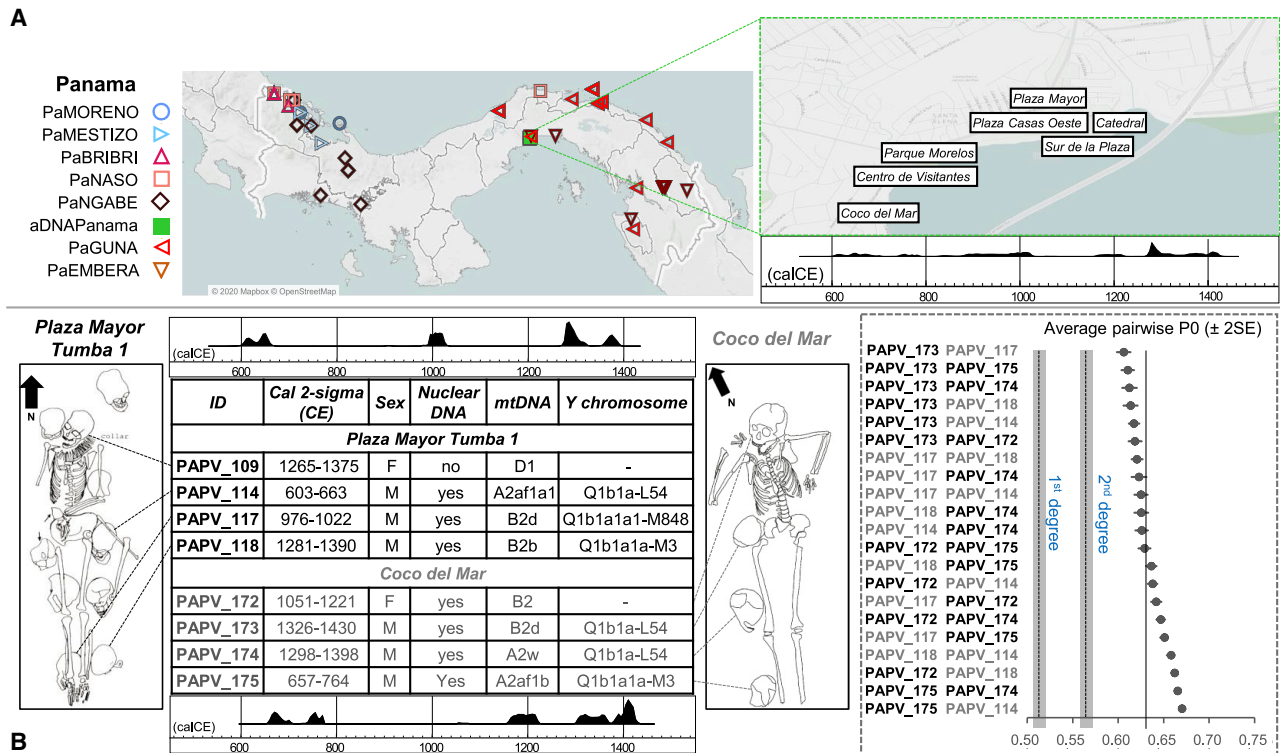


Figure 1. Geographic locations and time ranges of modern and ancient individuals sampled

(A) Map showing the geographic origin of the Isthmian individuals sampled; the inset represents the locations of the archaeological excavations. (B) Schematic drawings of Tumba 1 in the Plaza Mayor site of Panamá Viejo and the burial at Coco del Mar. The table reports mtDNA and Y chromosome haplogroup affiliations, molecular sex determination, and ^{14}C -calibrated dates (CE). The sum distributions of all ages combined are shown, separately for the two sites, above (Tumba 1) and below (Coco del Mar) the table. Calibration dataset was IntCal20. Calibration software was OxCal 4.4.2. The inset on the right shows no kinship relationships (values extracted from Table S2 with error bars indicating 2-fold Standard Errors, SE) among individuals buried together in Plaza Mayor Tumba 1 and Coco del Mar. IDs (and additional information) are indicated in black and gray, respectively.

(Figure 1B). Crania interred with prestigious individuals have been interpreted as evidence either of ancestor veneration or of human sacrifice with the ostentation of trophy heads (Mendizabal, 2004; Smith-Guzmán and Cooke, 2018). Arguments in either case draw on presumed (recent or ancestral) tribal and biological relationships. Using genome-wide data, we can now exclude any genetic relatedness among the individuals. Moreover, the two females exhibit different mtDNA haplogroups (D1 and B2, respectively) with respect to the surrounding male crania (A2af1a1, B2b, and B2d in Tumba 1; A2af1b, A2w, and B2d in Coco del Mar).

In combination with these genetic results, radiocarbon dates obtained for the pre-Hispanic individuals sampled from Panamá Viejo and Coco del Mar point toward a more complex and nuanced interpretation. The two female figures, PAPV109 (1265-1375 CE) and PAPV172 (1051-1221 CE), were interred with crania dated from 603 to 1,390 CE (2 sigma) in the first case and from 657 to 1,430 CE (2 sigma) in the second (Figure 1B). Hence, skulls spanning over 700 years, including the area's earliest and latest pre-Hispanic remains recovered to date, accompanied each of the main individuals. Seven of the skulls that accompanied PAPV109 belonged to individuals who pre-dated her by hundreds of years, and the other two were roughly contemporary. One of the crania buried with

PAPV172 belonged to a male individual who lived roughly 500 years before her, and the other two to male individuals deceased and buried over the subsequent 300 years. Drawing upon on Cueva as well as Guna ethnography (Castillero Calvo, 2017: pp. 26, 87, 281, 282, 476-478; Fernández de Oviedo, 1853: Vol. 2, pp. 125-154; Fortis, 2013), crania kept for hundreds of years or even deposited after the main interment probably pertained to enemy chiefs whose death in battle guaranteed their spirits' eternal repose. Their skulls may have provided sorcerers and healers, in this case female seers or *tequina*, a gateway to knowledge about enemies as well as the afterlife. These women entered the next world with the tools of their trade (the skulls), like other individuals interred in Panamá Viejo (a "musician" poised as if playing her instrument, and a seated adolescent with flint blades and stingray tail barbs) or mentioned in Spanish chronicles (farmers buried with corn) (Fernández de Oviedo, 1853: pp. 125-154). The heads of prestigious enemies obtained in warfare would have facilitated the seers' access to their strength and knowledge as well as their ability to communicate with other worlds. The different mtDNA lineages of these individuals might also support their origins from various pre-Hispanic groups, since we have found significant differences in the haplogroup distribution among the modern Indigenous populations analyzed

here (p value < 0.0001). Although the literature contains reference to wives, slaves, and loyal servants sacrificed with their chiefs (Fernández de Oviedo, 1853; Romoli, 1987), these burials illustrate a different practice. Their arrangement stands out among a great diversity of pre-Hispanic interments over a wide zone of the Panamanian Pacific, some of these including offerings in local pottery, metal, lapidary, shell, and bone work, and many burial modes, including bones deposited in urns or bundles (Martín, 2002a, 2002b). PAPV172's head was found beneath a ceramic offering, while five ceramic pots with offerings accompanied PAPV109, who also wore a necklace fashioned from thorny oyster shells (*Spondylus* spp.). Within the variety of pre-Hispanic burial patterns observed to date, individuals PAPV109 and PAPV172 appear unique on a local as well as a regional level.

Deciphering genomic variation on the Isthmus

The analyses conducted in this study facilitate a microgeographic and diachronic assessment of Indigenous autosomal variation in this strategic region. The characterization of the pre-colonial genetic histories is clouded by the impacts of colonization. This is evident in the distinctive genetic profiles that differentiate the current gene pool of the Panamanian groups as obtained by the ADMIXTURE analyses (Figures 2A, S2A, and S2B). The two groups that experienced a history of admixture, the self-identified “Moreno” and “Mestizo,” reveal large proportions of their genomes not derived from Indigenous peoples of the Americas. Both show a comparable proportion of ancestry predominant in Europeans (K2) and the component common to Africans (K3) is more prevalent in the “Moreno” group; the “Mestizo” are characterized by a component also identified in Asians (K4). The coexistence of different continental genomic ancestries is common in the Americas, due to complex admixture that started during the colonial period (Ongaro et al., 2019). In Panama, where European colonization began in 1,502 CE, this is particularly evident in the “Mestizo,” but it is also revealed by individuals who self-identified as Indigenous and genealogically unadmixed, showing variable amounts of African and European ancestries in their genomes, with the lowest average values in the Guna, followed by the Ngäbe.

The modern and ancient Isthmian individuals are also characterized by a specific Indigenous component, which has been identified considering only modern individuals (K6, Figure S2A) as well as with the addition of ancient individuals (K9, Figure 2A). This component drives the Isthmo-Colombian axis, depicted by the first (main) component in the principal-component analysis (PCA) of Indigenous groups (Figure 2B), which includes ancient and modern Panamanians together with the Cabécar from southern Costa Rica and two populations from northern Colombia (the Wayuu and the admixed Columbia [CLM]). The Indigenous groups from the pre-colonial Greater Chiriquí cultural area form two closely related western clusters (one with the Ngäbe and the other including Bribri, Naso, and Cabécar). The pre-Hispanic individuals group together in the middle of the Isthmo-Colombian genetic landscape and create a distinct branch in the outgroup f_3 statistics hierarchical tree, together with a few self-identified “Moreno” (Figure S3A), suggesting the integration of pre-Hispanic individuals into the forming multi-cultural colonial groups. The

genetic closeness of the pre-Hispanic individuals is possibly expected when considering the geographic proximity of the archaeological excavations but less expected when taking into account the radiocarbon dates, from 603 to 1,430 CE, thus revealing a genetic continuity for almost one thousand years (Table S1). The modern Indigenous populations from the Greater Darién area of cultural influence to the east of the region (Guna, Emberá, and northern Colombians) create distinct groups in the PCA plot, well separated from the Greater Chiriquí populations in the west.

The details of this genetic sub-structure (and heterogeneity) on the Isthmus became apparent by analyzing the nearly unadmixed Indigenous haplotypes (uA217 dataset) with fineSTRUCTURE (Figures 3A and S4A). Among the five Isthmian genetic clusters, four are specific to Indigenous Panamanian groups (PaNASO, PaNGABE, PaEMBERA, PaGUNA), while the Bribri individuals form a separate cluster (here called Western Isthmus) with the Cabécar from Costa Rica. The latter branch, together with Naso and Ngäbe, forms a macro-group that might be associated with the geographic region of the pre-colonial Greater Chiriquí cultural area. Genetically distinct are the Emberá and the Guna, suggesting a wider genetic variation in the Greater Darién cultural region. The Guna also show the highest level of similarity in intra- and inter-cluster comparisons (Figures 3B and S4B), analogous only to two Indigenous groups that experienced isolation events, the Amazonian Surui and Karitiana, and preserved an ancient Australasian-related ancestry, the so-called UPopY (Moreno-Mayar et al., 2018; Skoglund et al., 2015). We formally looked for UPopY variants in the Isthmus with f_4 statistics in the form f_4 (W/Panama, X/Mixe; Y/Australasia, Mbuti) without finding any significant sign of admixture or gene flow (Figure S5A). The same statistics were used to formally test the average correlation in allele frequency differences (mixture of ancestries) within the Isthmo-Colombian area (Figure 2C). This analysis provides statistical support to the genetic interactions (Z score often $< |3|$) in the western Isthmian area, eventually extended to Cabécar, Naso, and Ngäbe. On the other hand, it reveals close relationships among the Emberá and northern Colombians (CLM and Wayuu). Finally, the pre-Hispanic communities inhabiting the Pacific coast in the area of Panama City cluster together with the Guna compared with other Isthmo-Colombian populations (Z score $< |3|$), except for the Ngäbe.

Deciphering genomic connections outside the Isthmus

Previous studies (Gnecchi-Ruscone et al., 2019; Moreno-Estrada et al., 2013; Reich et al., 2012) have already provided hints of genetic patterns in the Isthmo-Colombian region, eventually extended to other groups that speak Chibchan languages. Here, we first confirmed that the previously discussed Isthmian component is also detectable in additional Chibchan-speaking populations genotyped with a different array (Arhuaco and Kogi from Colombia; Guaymí, Cabécar, Teribe, Bribri, Huëtar, and Maleku from Costa Rica) and that its highest legacy can be detected in the western Isthmian land bridge consisting of the present-day countries of Panama and Costa Rica (Figure S2C). We have now detailed patterns of genomic variation of the area's core population(s), represented by pre-Hispanic Isthmian individuals and modern Panamanians, underlining their unique features in the Americas' genetic landscape.

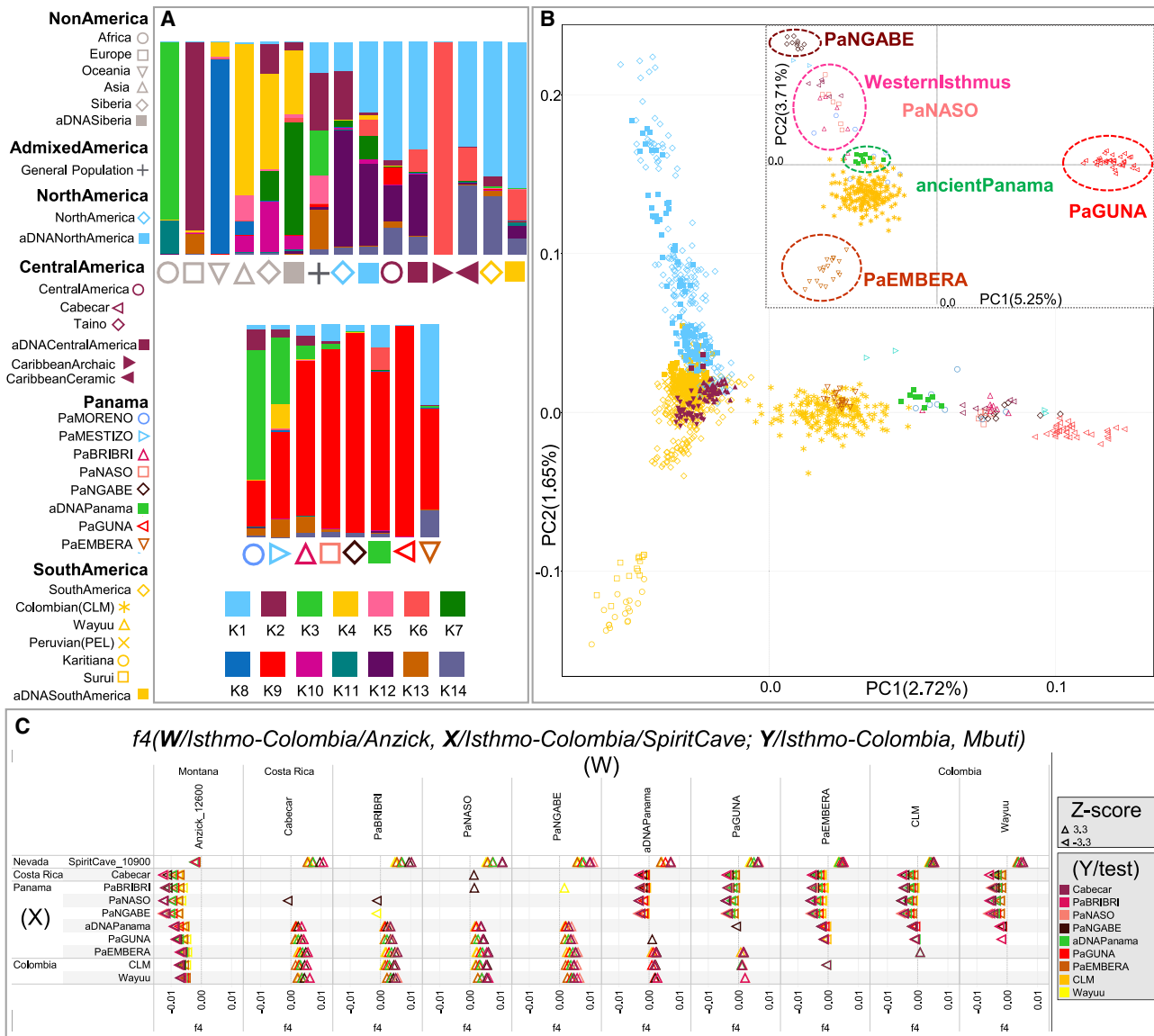


Figure 2. Overview of the genetic structure of ancient and modern Isthmian groups

(A) ADMIXTURE plot for $K = 14$; each bar shows the average ancestry proportion of individuals within the same group considering the rWD1560 dataset plus the American and Siberian ancient individuals.

(B) Indigenous American (IA) PCA analysis including the mIA417 dataset and ancient genomes projected onto uIA217 variability. The inset shows a specific Isthmo-Colombian PCA.

(C) f_4 statistics in the form $f_4(W/Isthmo-Colombia/Anzick, X/Isthmo-Colombia/SpiritCave; Y/Isthmo-Colombia, Mbuti)$ considering the uIA89 and mIA417 datasets plus ancient Isthmian individuals (all SNPs). The f_4 values are reported in abscissa. Each tested population (Y) is shown (with triangles pointing to X or W population) only when the initial conformation of the tree is rejected (p value ~ 0.001 , for Z scores $> |3.3|$), thus visually pointing to the closer population (X or W) in each comparison. A legend for symbols used in the paper is reported on the top left.

The f -statistics tests not only detected higher levels of shared genetic history between ancient and present-day groups of the Isthmian area extended to Costa Rica (Cabécar) and northern Colombia (CLM and Wayuu) in comparison with other ancient and modern populations (Figures 4 and S3B) but also revealed different levels of shared ancestries in the Isthmian groups (Figure S5B). The same pattern has been detected in the neighbor-joining tree (based on outgroup f_3 statistics and rooted

with an ancient Beringian genome; Figure S3C) of ancient and modern IA groups. This graph identifies an early Isthmo-Colombian branch with different sub-branches. This and the additional IA branches (with a geographic pattern) largely overlap with the haplotype-based clusters identified in the American-wide uIA217 dataset (Figure 3A), where in the absence of data from ancient individuals, the distinctiveness of the Isthmo-Colombian area in the modern America genomic structure remains evident.

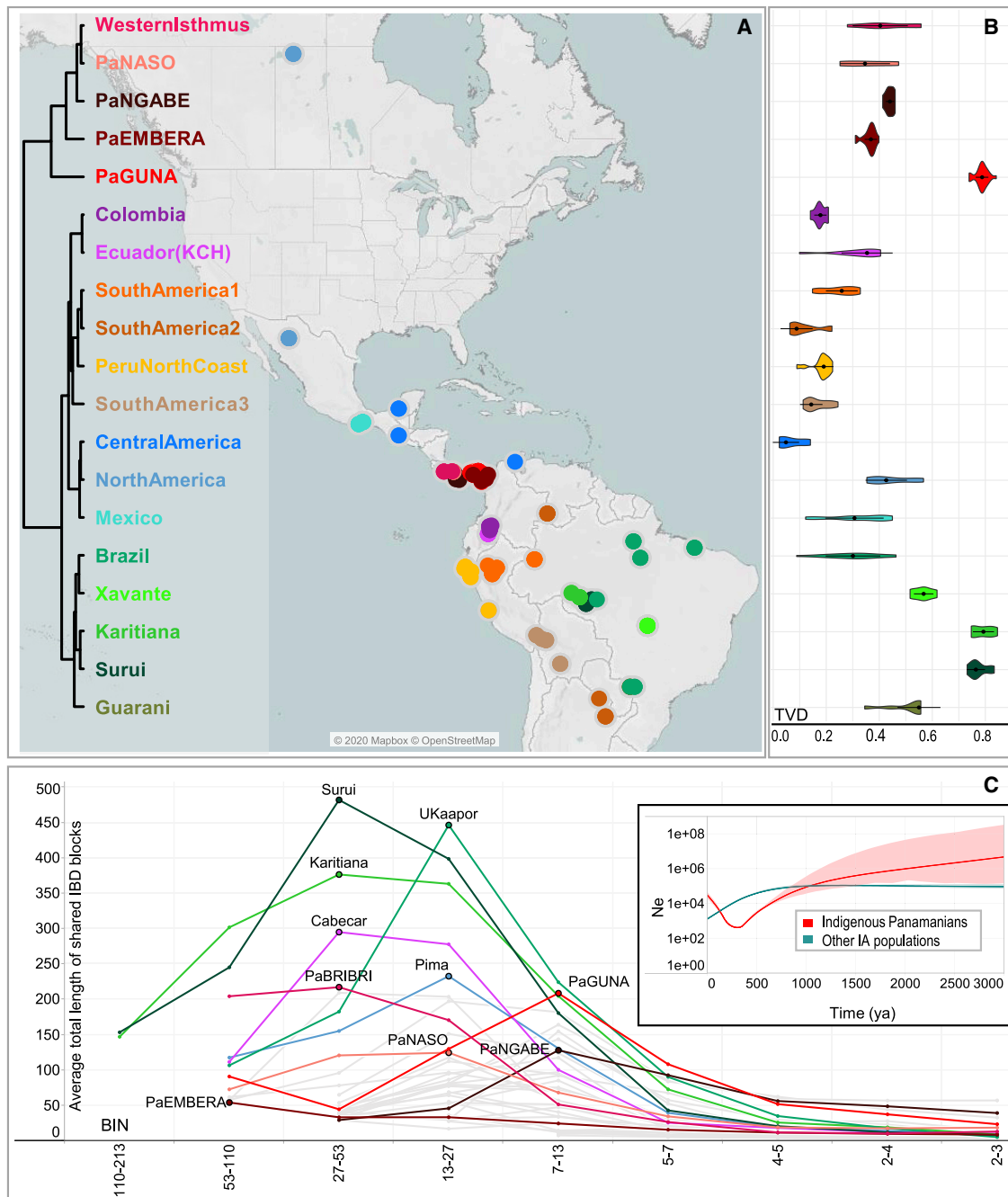


Figure 3. Population genetic structure as revealed by haplotype analysis of modern Panamanian and IA populations
 (A) fineSTRUCTURE unrooted dendrogram showing the 19 identified Indigenous clusters and the geographic distributions of the individuals in the nearly unadmixed IA (uIA217) dataset.
 (B) Violin plot showing cluster self-copy lengths (fragments copied from members of their own cluster) in the uIA217 dataset; higher values are for more isolated groups.
 (C) Density of the intrapopulation average total length of shared IBD blocks, considering nine bins of IBD lengths in the Panamanian and non-Panamanian Indigenous groups of the uIA217 dataset. The inset shows the estimation of changes in effective population size (N_e) over time based on IBD segments with a minimum threshold of 2 cM (even if estimates older than ~1,000 years should be considered with caution) and considering a generation time of 25 years.

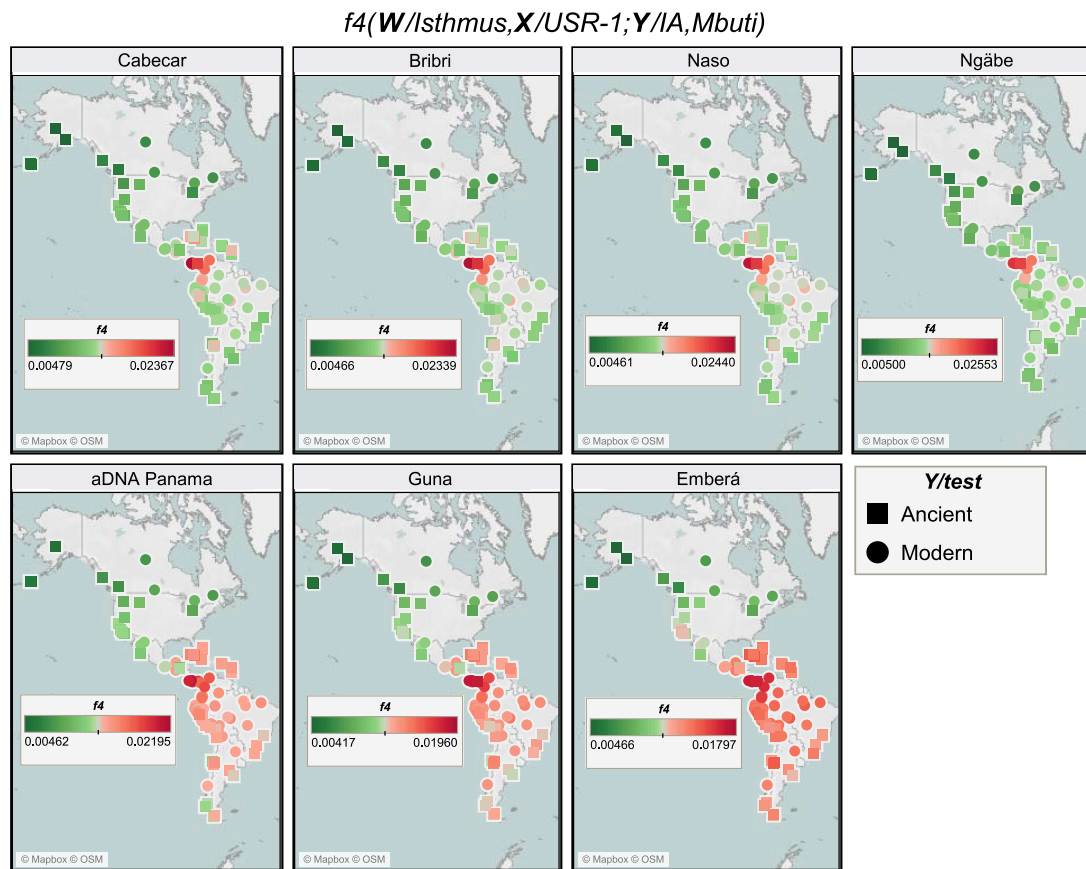


Figure 4. Heatmaps based on f_4 statistics to compare the differential relationships between the Isthmian groups and other ancient/modern IAs

f_4 statistics in the form $f_4(W/Isthmus, X/USR-1; Y/Ancient \text{ and } Modern \text{ IA}, Mbuti)$. Each tested population (Y) is represented by squares (ancient) and circles (modern) with colors proportional to f_4 values; Z score always >3.3 .

A pre-Hispanic origin of the Isthmo-Colombian distinctiveness is suggested by our analysis of effective population size based on the identical-by-descent (IBD) segments. A reduction in the population size of the Panamanian groups probably began during pre-colonial times (~ 1 kya; inset of Figure 3C), thus before the average time of the other IA population groups taken together. This trend is mainly driven by the Guna, who on the other hand show unreliable population size (N_e) values when considering short fragments (<8 cM, Figure S4C). Therefore, we repeated this analysis without the Guna, confirming an earlier beginning of the N_e reduction in the Panamanian IA groups that became steeper in colonial times (Figure S4D). Finally, N_e estimates based on IBD longer (and more reliable) fragments (>8 cM) allowed us to confirm this trend for the Guna (Figure S4E). Three factors may have contributed to this demographic pattern: (1) a decrease in the population size that started before European contact; (2) a less intensive impact of European colonialism, as also suggested by the lower peaks of IBD bins for most Panamanian groups; and (3) an earlier and steeper demographic recovery after contact than in other IA populations, especially evident in Guna and Ngäbe who show an enrichment of shorter IBD segments, around 7–13 Mb, and then

a fast decrease of IBD blocks (Figure 3C). All explanations are plausible and not mutually exclusive. Before European contact, cultural changes in the Panama Bay region may have accompanied migrations and demographic shifts (see “Panama: archaeology and history” and, in the STAR Methods section, “Insights into pre-Hispanic Panama”). This possibility finds support from other genetic analyses that showed a high level of genomic drift shared by the Isthmian Indigenous groups, including the ancient individuals sampled, on both allele frequencies and haplotypes (see PCA plots and all the trees), as well as from the comparison of the IBD fragments shared among the Panamanian groups and with other IA populations (Figure S4F). The latter analysis reveals ancient interactions (from at least 2,500 years ago) within the Isthmo-Colombian area, much stronger and temporally extended among the western populations (Cabécar, Bribri, and Naso) currently living in the geographic region associated with the pre-colonial Greater Chiriquí cultural area. On the eastern part of the Isthmus, the Guna also show a number of short (older) blocks shared with the Maya from Mexico, while the Emberá share shorter blocks with South American populations. Thus, the former probably received ancient genetic inputs from the north, while the

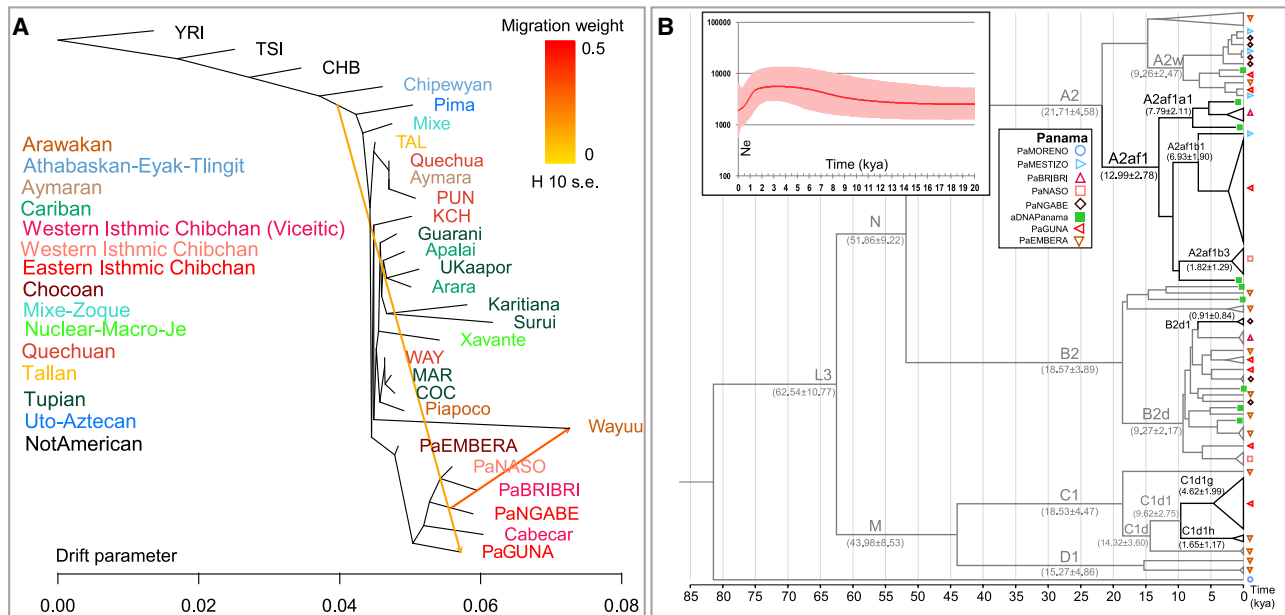


Figure 5. Schematic phylogenetic trees based on genome-wide and mtDNA data

(A) Inferred maximum likelihood tree built with TreeMix on the unadmixed dataset uIA89 allowing two admixture edges (migration events). Population groups are colored according to linguistic/geographic affiliation. Horizontal branch lengths are proportional to the amount of genetic drift that has occurred on the branch. Migration arrows are colored according to their weight.

(B) Bayesian phylogenetic tree of ancient and modern mitogenomes from Panama belonging to IA founding haplogroups. It was rooted on an L2c2 mitogenome from a “Moreno” individual. The Bayesian age (mean value with standard deviation) is shown for relevant branches. Black lines highlight Isthmo-Colombian-specific branches. The inset shows the Bayesian skyline plot (BSP), based on complete mitogenomes, displaying changes in the effective N_e through time considering a generation time of 25 years.

latter admixed with external southern sources. The Guna also show a direct connection with the ancestors of North and Central American populations in the TreeMix maximum likelihood (ML) tree when two migration edges (gene flows) are added (Figure 5A). Such an ancient legacy is also confirmed by the Panamanian mtDNA tree. The most-represented haplogroups among ancient and modern Panamanian mitogenomes belong to the four main pan-American founding lineages (A2, B2, C1, and D1; Figure 5B). We also identified four Isthmo-specific sub-branches, the most represented one (A2af1) is dated at 15.82 ± 4.09 kya (Figure S1A). Finally, the Bayesian skyline plot (BSP) of Panamanian mtDNAs shows an increase in population size starting in the early Holocene (~ 10 kya) (inset of Figure 5B).

A previously undescribed ancestry among ancient Indigenous peoples of the Americas?

To further understand the peculiarities of the Isthmo-Colombian populations within the context of the most updated archaeogenomic scenario of non-Arctic America (see Introduction), we used f_4 statistics, which controls for possible biases deriving from population-specific drift, to compare ancient individuals and contemporary Indigenous groups to the individuals we sampled. As expected, the Isthmus shows an excess of allele sharing with modern and ancient Indigenous populations from Central and South America when compared with Ancestral Beringia (USR-1, Upward Sun River, Alaska, ~ 11.5 kya) and NNA

(represented by ASO, Ancient Southwestern Ontario, ~ 4.2 kya) genomes, but this picture is more intricate when dealing with the SNA-related ancient genomes (Table S4). The affinities between the (Y/tested) Isthmian populations and other Indigenous (X) groups are significantly stronger in relation to Anzick-1 (Montana, ~ 12.8 kya) than to Spirit Cave (Nevada, ~ 10.9 kya) (W individuals; Figure 6A), suggesting that Isthmian populations are related to Spirit Cave as much as to other Indigenous groups, while Anzick-1 is an outgroup to them. We directly tested the relationships of Isthmian and other Central/South American populations with Anzick-1 and Spirit Cave, highlighting a differential trend that becomes significant with a higher molecular resolution power, i.e., more single-nucleotide polymorphisms (SNPs) (Figures 2C and S5C). Moreover, the Isthmus seems more closely related to Spirit Cave than to Anzick-1 in comparison with Ancestral Beringia.

Such genomic differences are confirmed when moving southward in Central America (Table S4) and particularly for the early ancient genomes excavated on the southern continent. The Pacific coast populations (Los Rieles, Chile, ~ 10.9 kya) exhibit greater affinity to Spirit Cave, while the ancient genomes from the Atlantic side show the same pattern as Anzick-1 when considering individuals older than ~ 7 kya (Lagoa Santa, Brazil, ~ 10.4 kya). These distinctive signals persisted up to about 7 kya, when they were probably erased by a major population turnover in South America (Moreno-Mayar et al., 2018; Posth et al.,

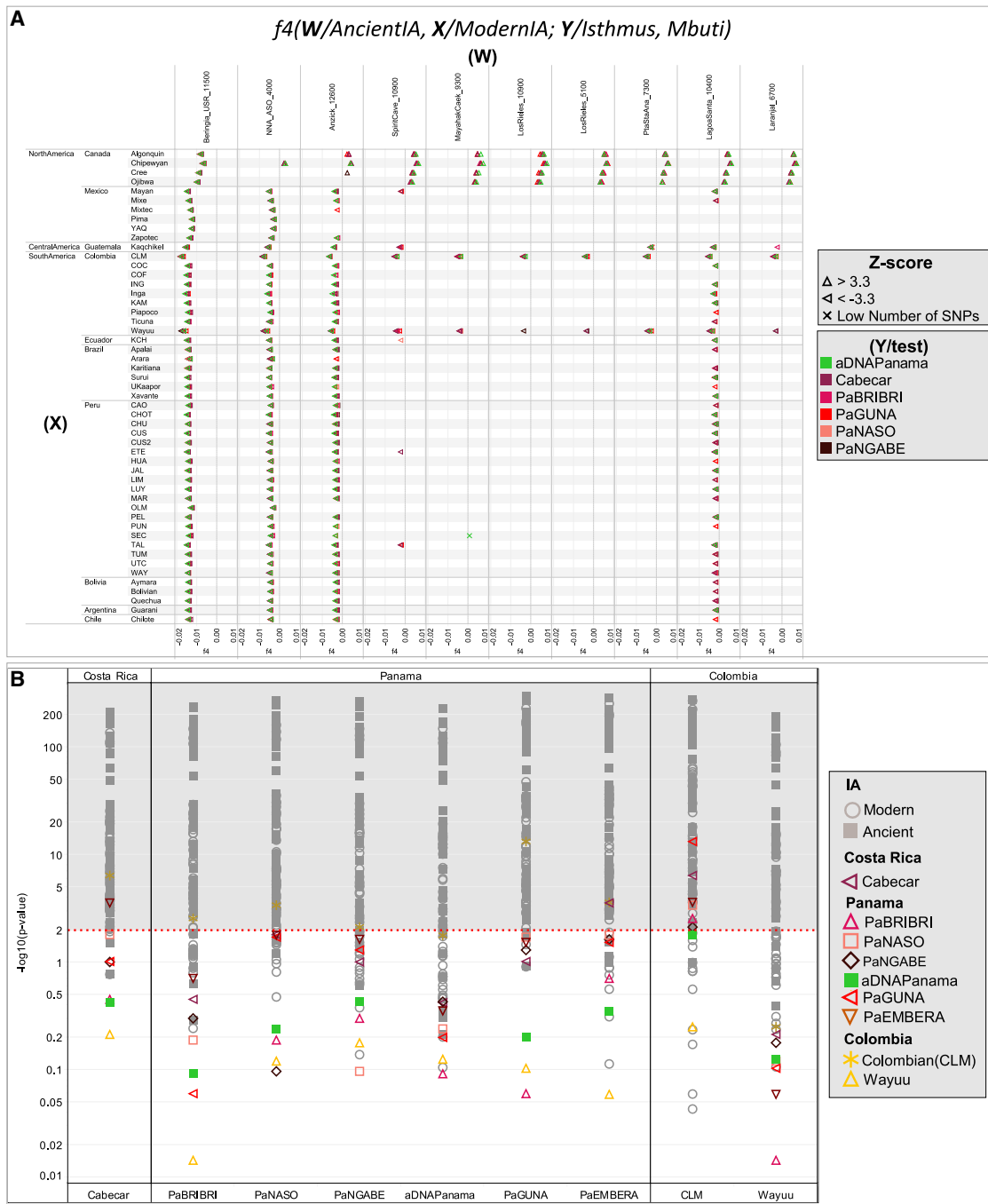


Figure 6. f_4 statistic tests on Isthmian and other IA groups and minimum number of ancestral sources

(A) f_4 statistics in the form $f_4(W/AncientIA, X/ModernIA; Y/Isthmus, Mbuti)$ on uiA89, miA417, and ancient genomes considering only transversions. Only subgroups of meaningful ancient genomes were considered (see Table S4 for comparisons with the entire ancient dataset). The Emberá group was excluded due to a significant degree of admixture detected in the individuals. Each tested population (Y) is shown (with triangles pointing to X or W population) only when the initial conformation of the tree is rejected ($p \sim 0.001$, when the Z score is $>|3.3|$), thus visually pointing to the closer population (W or X) in each comparison.

(B) We used qpWave to compare (in pairs) ancient Panama and present-day Isthmian groups with all IA populations (considering rank 1). Outgroups were kept to the minimum and chosen to represent different IA ancestries identified here (STAR Methods) and in other papers. Rank 1 refers to a model in which all paired populations fit as derived from two ancestral sources, relative to the outgroups. A p value > 0.01 (< 2 in $-\log_{10}$ scale, dotted red line) means pairs that could be explained by a single independent source.

Fit	Obs	Diff	Std.error	Z-score
0.000000	0.002515	0.002515	0.001102	2.283
Score 9.378				
Without UPopI, Z =4.255, Score 9.984				
Mixe(UPopA) -> Guna(UPopI), Z =3.401, Score 9.582				
Anzick-1 -> Spirit Cave, Z =3.160, Score 9.630				

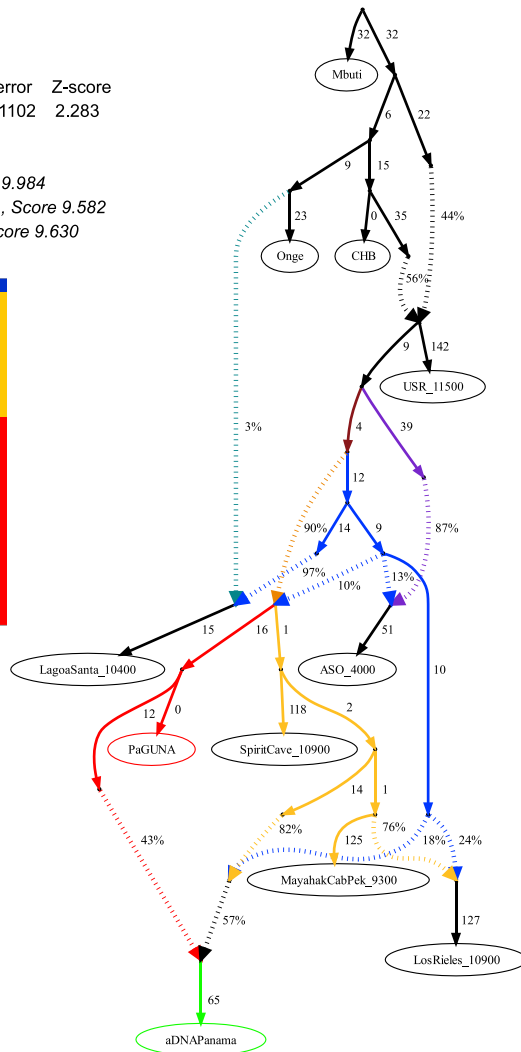
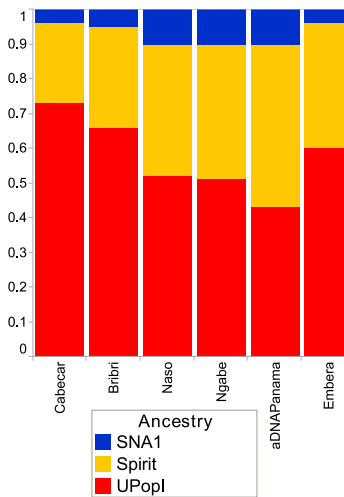


Figure 7. Admixture graph modeling ancestries and affinities of Isthmian groups in America

Best fitting f -statistics-based admixture graph optimized using qpGraph. We modeled the genetic history of ancient Isthmian individuals and the Guna directly linked to ancient IA genomes representative of the SNA ancestries. At the top, we show the f_4 statistics with the worst Z score after optimizing the model. Statistics on alternative models are also listed (see Figure S7 for further details). Numbers to the right of solid edges represent optimized drift parameters and percentages to the right of dashed edges represent admixture proportions. Different colors indicate the specific ancestries discussed in the text. The bar chart shows different ancestry proportions in ancient and modern Isthmian groups (except for Guna) estimated with qpGraph on the final tree (Z score always < |2.5|).

tries than the average of the one shared by other IA populations (Figure S3D). Therefore, we modeled admixture graphs looking for the most plausible origin of the Isthmian component (Figures S6 and S7; see also the specific section of STAR Methods for further details). The best supported topology successfully tested the hypothesis that the ancestral gene pool of the Isthmo-Colombian area, here represented by pre-Hispanic Panamanians, derives from a local admixture between different ancestral components (Figure 7). One derives from the differential mixture of two ancestries, SNA1 and SNA2, that in turn stem from an ancestral SNA source. This scenario is strongly suggestive of the first split between SNA and NNA occurring in

2018), facilitated by a widespread population decline due to mid-Holocene climate changes (Riris and Arroyo-Kalin, 2019).

The results of previous analyses revealed that Isthmian and non-Isthmian IA populations are differentially related to available Pleistocene individuals, suggesting the contribution of different sources. To test whether the Isthmian and non-Isthmian groups derived from the same or distinct ancestral populations, we used qpWave (Patterson et al., 2012), which estimates the minimum number of sources necessary to explain the observed genetic composition of population groups. Significance values are consistent with pairs of Isthmian and non-Isthmian groups deriving from at least two separate streams of ancestry, as attested by rank 1 p value < 0.01 in most comparisons, especially for the Guna (Figure 6B). This finding demonstrates that the distinctiveness of the Isthmo-Colombian area cannot be explained by genetic drift alone, as recently inferred in other population contexts (Nägele et al., 2020). The Guna also show lower values (mostly <25th percentile) of shared genetic history with ancient genomes representative of well-known Indigenous ances-

Beringia, thus further north than generally proposed (Waters, 2019). The NNA ancient individual in Figure 7, ASO (~4.2 kya), results from an admixture between NNA and SNA1. We could not identify an unadmixed proxy for the NNA ancestry among the available modern and ancient individuals (Figure S6H), but NNA does not seem to be involved in shaping the Isthmian genomic pool. Founding populations carrying the SNA1 ancestry probably took part in an early peopling of the double continent passing through the Isthmus and leaving signals on both sides of South America, as attested by two of the most ancient genomes, Lagoa Santa in Brazil (~10.4 kya) and Los Rieles in Chile (~10.9 kya). The former also confirms a few traces of UPopY, the UPop of Australasian ancestry, which was previously proposed to have contributed to the early peopling of South America (Moreno-Mayar et al., 2018; Skoglund et al., 2015). On the other hand, only Los Rieles shows significant inputs of the SNA2 ancestry, which moved later or slower than SNA1 through the Americas, admixing multiple times with the first settlers along the way, as demonstrated by the ancient admixed genomes of Spirit Cave in North

America and Mayahak Cab Pek (Belize, ~9.3 kya) and the ancient Isthmians (this study) in Central America. Once SNA2 reached South America, it probably left a stronger contribution on the Pacific side, as suggested by Los Rieles and supported by the differential pattern depicted by the f_4 statistics (Table S4). However, to fully explain the genetic variation of pre-Hispanic Panama, we need to consider an additional ancestry: an ancestral unsampled population of the Isthmus (UPopI; Figure 7), which is still unrepresented in the ancient dataset, but left its strongest traces in the contemporary Guna. UPopI parallels the Spirit Cave branch of SNA2, testifying to a long-shared drift between the pre-Hispanic Guna and the ancient Isthmian (including pre-Hispanic) individuals sampled. This best-fitting model was also checked without considering UPopI and replacing Spirit Cave with Anzick-1 and Guna with Mixe, previously used to identify UPopA (Moreno-Mayar et al., 2018), without finding any statistically supported graph (Figures S7D–S7F). Therefore, it is supported that Anzick-1 and Spirit Cave might represent different ancestries and UPopI is a still unsampled population distinct from UPopA. UPopI likely originated in the north during the late Pleistocene, as attested by the age (~15 kya, Figure S1A) of the entire mtDNA haplogroup A2af1 that probably represents its mitochondrial legacy, and expanded more than 10 kya in the Isthmo-Colombian area (according to mitogenome data; inset of Figure 5B). These ancestries left different traces in pre-Hispanic Isthmian individuals and contemporary Panamanian groups (Figure 7), and only the presence of UPopI provides significant support for our final model. It also explains the ancestral component, already seen in the ADMIXTURE analyses, which is geographically restricted to the Isthmo-Colombian area and prevalent in the Guna, where it was probably maintained by a high level of isolation. Finally, this specific ancestry could explain the “archaeogenomic distinctiveness” of the Isthmo-Colombian area within the genomic landscape of IA groups.

DISCUSSION

Our work enriches the IA genomic database with autosomal data from cultural groups of Panama and ten low-coverage pre-Hispanic genomes obtained from human remains excavated in the tropical area of Panama City on the Pacific Ocean. The ancient genetic profiles from Panamá Viejo and Coco del Mar sites, radiocarbon dated from 603 to 1,430 CE, confirm similarities in the gene pool of this pre-Hispanic population(s), suggesting common origins. The diachronic comparison with population groups presently living in Panama allowed us to identify genetic similarities with the Guna and Ngäbe and suggestive connections with some admixed individuals, implying that, in the wake of the conquest, there was extensive gene flow. A genetic sub-structure has been identified in the entire Isthmo-Colombian region, with a macro-group encompassing the Cabécar, Bribri, Naso, and Ngäbe, who currently live in the pre-colonial Greater Chiriquí cultural area. A wider genetic variation characterizes eastern Panama, here represented by pre-Hispanic individuals from Panamá Viejo and Coco del Mar plus Guna, Emberá, and northern Colombians. Our analyses suggest that pre-Hispanic demographic changes and isolation events, evident in the Guna, contributed to create the genetic structure currently seen in the re-

gion. Moreover, through allele frequency analyses and haplotype-based reconstructions, we describe the presence of a specific axis of Indigenous genetic variation in the Americas, which is typical in the Isthmo-Colombian area and possibly extended to other Chibchan-speaking groups. This component was present not only among pre-Hispanic Isthmian individuals but also strongly characterizes current Panamanian groups, particularly the Guna, surviving both pre-colonial demographic fluctuations and the genetic bottleneck (and admixture) caused by colonialism.

The detection of this component has an impact that expands far beyond the Isthmo-Colombian area and the ancestry of its past and current inhabitants. The following clues point to the scenario that it arose in the late Pleistocene: (1) the pre-Clovis age of the Isthmian-specific mtDNA haplogroup A2af1, (2) the internal structure that emerges when only the Indigenous genome-wide variation is analyzed, (3) the longer shared genetic history among Isthmo-Colombian populations with respect to other Indigenous populations, and (4) the differential relationships with Pleistocene individuals from North America. Next, to identify its ancestral source(s), we built a statistically significant model that explains this Isthmo-Colombian component as a local admixture of different ancestries of northern North American origin. At least two SNA ancestries, SNA1 and SNA2, differentially associated to available Pleistocene genomes, should be considered, as well as an additional Isthmian-specific ancestry. The latter requires the contribution of UPopI, which stemmed from the same source (SNA2) that contributed to the pre-Clovis groups with Western Stemmed technologies associated with Spirit Cave and, according to mitogenome data, expanded within the Isthmo-Colombian area during the early Holocene.

The ancestral admixtures described here were probably bound to now-submerged archeological sites on the Pacific coast of the Isthmus. Nevertheless, the genomes of the pre-Hispanic individuals from Panamá Viejo and Coco del Mar attest to these events, and the site of Vampiros-1 (initially named Cueva de los Vampiros), the only Pleistocene site on the lower Isthmian land bridge that contains cultural, but not human, skeletal remains, provides further archeological support. Vampiros-1 shows evidence of both Clovis and Fluted Fishtail Point lithic traditions indicating that hunter-gatherers of extra-Isthmian origin were on the lower Isthmus 13.2–11.7 kya with specific composite weaponry and cutting/scraping tools (Ranere and Cooke, 2020). Our model also fits well with recent archeological records from both sides of the Isthmo-Colombian area. Archeological findings in southern North America report early peopling as far south as central northern Mexico around the Last Glacial Maximum (LGM) (Ardelean et al., 2020) and more widespread settlements in warmer pre-Clovis times (14.7–12.9 kya) (Becerra-Valdivia and Higham, 2020). The cultural heterogeneity observed among the oldest reliable pre-Clovis archaeological sites of South America (dated 15.1–14.0 kya) along the Pacific coastal zone (Huaca Pietra in Central Andes; Monte Verde II in South Andes) (Dillehay et al., 2017) and in the Pampas (Arroyo Seco 2) (Politis et al., 2016) can be explained considering a deeper chronological time (between 16.6 and 15.1 kya) for the Isthmian crossing that led to the initial peopling of South America (Prates et al., 2020).

The preservation effect of an ancient legacy in “outlier populations,” such as the Guna, recalls that of Sardinians and Basques in Europe (Achilli et al., 2004; Chiang et al., 2018; Novembre et al., 2008; Olivieri et al., 2017; Palencia-Madrid et al., 2017). In the European context, Sardinians maintained the most evident traces of the early European Neolithic farmers (Lazaridis et al., 2014, 2016; Raveane et al., 2019). Among Indigenous peoples, some Amazonian groups, which match the very high internal similarities of the Guna, have preserved the specific ancestry of a UPop linked to Australasia (Moreno-Mayar et al., 2018; Skoglund et al., 2015). In the Isthmian context, demographic events connected to both pre-Hispanic and colonial times contributed to differentially retain and further shape the IA ancestries.

Limitations of study

The models reported in this study are based on 12 ancient low-coverage genomes from two archeological sites and genome-wide data from 74 unrelated modern Panamanians. These data provide a suggestive baseline for future interdisciplinary studies on the Isthmo-Colombian crossroads. High-coverage genomic data, from a wider time frame (since early Holocene to colonial times) and other archeological sites (across the entire Isthmo-Colombian area), as well as whole genomes from present-day individuals, are needed to continue to refine the region’s genetic history. This additional work, with more statistical power and higher molecular resolution, will be essential to further detail the genetic patterns (and ancestries) that we have identified in the Panamanian population(s) and to reconstruct variation in population sizes over time.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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● ADDITIONAL NOTES

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cell.2021.02.040>.

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AUTHOR CONTRIBUTIONS

J.R., T.M., I.H.-M., and J.G.M. excavated archaeological sites, provided or acquired ancient samples, and contextualized the archaeological findings. M.T. and U.A.P. performed modern sample collections supervised by J.M.P., J.M., O.S., and A.A. R.F. and C.K. produced and analyzed radiocarbon data. M.R.C., H.L., and A.M. performed aDNA laboratory work supervised by M.L., D.C., R.S.M., and A.A. M.R.C., A.R., and A.G.-C. produced modern genome-wide data supervised by A.S. and A.A. M.R.C., A.R., N.R.M., G.C., L.O., C.L.S., and M.L. analyzed ancient and modern genomic data supervised by F.M. and A.A. M.R.C., N.R.M., G.L., and G.S.G. produced and analyzed mitogenome sequences supervised by A.O., A.T., and A.A. M.R.C., A.R., G.C., and V.G. classified Y chromosome data supervised by O.S. G.H., F.B., C.C., L.P., and M.M. provided technical and computational resources. M.R.C., B.A., and A.A. wrote the original draft with major inputs from R.C., R.S.M., A.T., and J.G.M. All authors discussed the results and contributed to the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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