Two contemporaneous mitogenomes from terminal Pleistocene burials in eastern Beringia

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Pleistocene residential sites with multiple contemporaneous human burials are extremely rare in the Americas. We report mitochondrial genomic variation in the first multiple mitochondrial genomes from a single prehistoric population: two infant burials (USR1 and USR2) from a common interment at the Upward Sun River Site in central Alaska dating to ∼11,500 cal B.P. Using a targeted capture method and next-generation sequencing, we determined that the USR1 infant possessed variants that define mitochondrial lineage C1b, whereas the USR2 genome falls at the root of lineage B2, allowing us to refine younger coalescence age estimates for these two clades. C1b and B2 are rare to absent in modern populations of northern North America. Documentation of these lineages at this location in the Late Pleistocene provides evidence for the extent of mitochondrial diversity in early Beringian populations, which supports the expectations of the Beringian Standstill Model.

Pleistocene burials | ancient mitochondrial DNA | paleogenomics | peopling | Americas

The colonization of the Western Hemisphere has been of interest to scholars since 1590, when Jose de Acosta postulated a northeast Asian origin of the indigenous populations of the Americas (1). Both the archaeological (2, 3) and genetic (4–10) records consistently indicate a primary entry point from Asia to the Americas via the Bering Land Bridge, sometime during the Late Pleistocene. However, there are unfortunate lacunae in both records. The archaeological record indicates a relatively late (<14–16 kya), rapid colonization event following the Last Glacial Maximum. This temporal scale supports the clear northeastward geographical expansion of late Upper Paleolithic (Diuktai) populations from southern and central Siberia to Beringia after 16 kya (5). However, archaeological evidence is accumulating that shows people had penetrated parts of North and South America before 13,250 cal B.P., the earliest date associated with Clovis, the first widespread cultural tradition in North America (2–5, 11).

The genetic record is equally problematic. Continental scale analyses of genetic variation rely heavily on Central and South American population data, as well as data from Arctic populations (6–9, 12, 13). Few data exist for North American populations south of the Arctic. Recent surveys of contemporary genetic variation in the Americas are consistent with a period of population isolation during which the distinctive composition of Native American genomes differentiated from ancestral Asian genomes, followed by a rapid colonization; this scenario has been deemed the "Beringian Standstill Model" (6, 7, 10). How early the Native American gene pool diverged remains uncertain, but estimates of up to 30 kya have been postulated (5, 6, 10, 12, 14, 15). Most geneticists argue for at least a several thousand-year period of isolation and genetic differentiation in Beringia before a southward dispersal, despite the absence of supporting archaeological evidence (2, 4, 5, 10). Recently, Raghavan et al. (15), using genome-wide low-coverage data, suggested the dates of this isolation began no earlier than 23 kya and lasted no longer than 8,000 y (15).

Ancient DNA (aDNA) samples from early inhabitants of the Americas would be important for linking the modern genetic and archaeological records (16), but few exist. The Mal'ta child from South Central Siberia indicates an early origin (>24 kya) of some signal of Native American ancestry (9), but although a few Pleistocene-aged remains have been recovered in central North America (below the Laurentide Ice Sheet) or along the Northwest Coast, no similarly aged Beringian human remains have previously been available for genetic comparison. Very few Late Pleistocene (>10,000 cal B.P.) individuals have yielded mitochondrial genetic (mtDNA) data, although we highlight the seven sites with ancient human remains dating to >8,000-y-old that have been characterized for mtDNA lineages: Hoyo Negro, Mexico (17); Anzick, MT (18); Kennewick, WA (19); On-Your-Knees Cave, AK; Wizard's Beach, NV; Hourglass Cave, CO; and, indirectly through coprolite analysis, Paisley Cave, OR (the last four are reviewed in ref. 20) (Fig. 1).

In 2011 Potter et al. (21) reported on the discovery of a cremated 3-y-old child from a residential feature at Upward Sun River (USR) in eastern Beringia dating to 11,500 cal B.P. Additional excavation at this deeply stratified and well-dated site (22) recently yielded two additional infant burials (Fig. 1) (USR1 and USR2) (23). A series of radiocarbon ages securely date the three individuals between 11,600 and 11,270 cal B.P. (23). Based on dental and osteological aging methods, USR1 represents a late preterm fetus, and USR2 likely died within the first 6 wk of life (23). The proximity of these three burials, their context within the same feature, and radiocarbon analyses presented in Potter et al. (23) strongly suggest that all three

Significance

Beringia gave rise to the first Western Hemisphere colonists, although the genetic characterization of that source population has remained obscure. We report two mitogenomes from human remains within Beringia, with an age (∼11,500 cal B.P.) that postdates the end of the initial colonization by only a few millennia. The mitochondrial lineages identified (B2, C1b) are rare to absent in modern northern populations, indicating greater genetic diversity in early Beringia than in modern populations of the region. The antiquity and geographic location of these two burials, and the combined genomic and archaeological analyses, provide new perspectives on the link between Asia and the Americas, and the genetic makeup of the first Americans.

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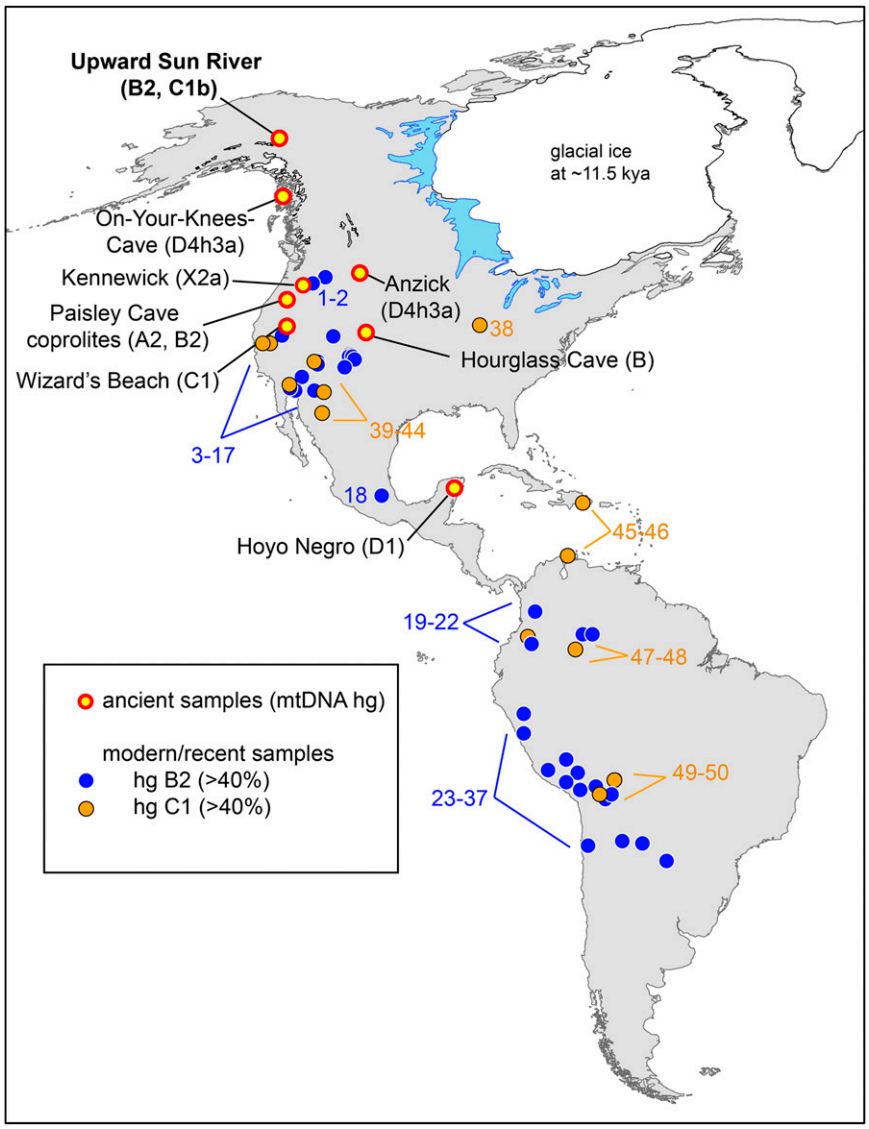

Fig. 1. Geographic map of reported Native American populations with >40% C1 or B2 haplogroup frequencies, as well as locations of archaeological sites discussed. The locations of the Upward Sun River site, as well as the seven previously reported archaeological sites dated at >8,000 y B.P. with successfully genotyped human mitochondrial DNA lineages, are listed on the map (with reported haplotypes). Reported populations of ≥20 individuals with ≥40% C1 (yellow) or B2 (blue) are shown. Populations and frequencies specific to this figure (referenced by numbers 1–50) are available in the *SI Materials and Methods*.

burials represent nearly contemporaneous events, and that the three individuals were members of a single population.

We attempted to extract and sequence the mitochondrial genomes from these three Late Pleistocene burials. From burnt bone fragments of the cremated infant and well-preserved samples of the petrous portion of the parietal bone, DNA was extracted using a silica-based method and attempts were made to Sanger sequence three overlapping fragments of the mitochondrial hypervariable region 1 (HVR1). From USR1 and USR2, all three HVR1 fragments were successfully amplified, and from the cremated infant only one fragment amplified, albeit inconsistently. DNA samples and applicable blank controls from USR1 and USR2 were converted to Ion Torrent Ion Plus Fragment libraries with laboratory-unique barcodes. We targeted the mitochondrial genomes by hybridization capture (24) and sequenced the libraries on two P1 chips with an Ion Proton System (Life Technologies). This is one of the first examples of the Ion Torrent technology applied to aDNA.

Results

From 58.7 and 55.8 million sequencing reads, 20,004 and 32,979 unique mtDNA reads (MAPQ ≥ 30) from USR1 and USR2, respectively, were mapped to the human mtDNA reference (Table S1). We used the Torrent Suite analytical pipeline to take advantage of flow space information, base recalibration, read realignment, and an Ion-optimized mapping (tmap) and duplicate filtering approach. This pipeline also allowed variant calling with the Torrent Variant Caller (TVC), providing a range of variant quality metrics identical to current best-practices approaches for next-generation sequencing of modern samples. This pipeline is optimized for Ion Torrent reads, unlike most methodologies currently used in the aDNA literature.

Sequencing of the enriched mtDNA from samples USR1 and USR2 resulted in 100% coverage of the mtDNA genomes with average read depths of 117× (geometric mean of 97×) for USR1 and 195× (geometric mean of 180×) for USR2 (Fig. S1). Mean read lengths for the two samples were 98 and 99 bp. Contamination estimates were made by dividing the reference allele counts at called variants by the total coverage from the TVC output; contamination rates were estimated at 3.5% and 4.9% for the two samples, respectively. Maximum parsimony (MP) analysis of SNPs and insertion/deletions (indels) in the full genomes indicated membership in mtDNA lineages C1b (USR1) (Fig. 2*A*) and B2 (USR2) (Fig. 2*B*). The mtDNA genome of USR1 had a private variant in the form of SNP C16292T. The B2 lineage carried by USR2 revealed a single back mutation at nucleotide position 3547 to an ancestral adenine. A subset of called variants, in addition to the previously typed HVR1, were validated by Sanger sequencing.

From the initial Torrent Suite bioinformatics pipeline we observed an irregular pattern of DNA damage expected from aDNA samples (Fig. S2). The 5′ ends of these reads had unexpected low quality base calls, likely from our custom adapters lacking a spacer sequence after the barcodes, and we were not able to investigate 3′ damage patterns. We initiated an alternative pipeline for reads from both Ion P1 chips: we performed additional read trimming for adapter sequence, length (30–120 bp), and quality, and we remapped (tmap) without 3′ clipping. Following this alternative pipeline, 21,140 and 22,951 mtDNA reads at MAPQ ≥ 70 mapped to the mtDNA genome from USR1 and USR2, respectively (Table S2). One-hundred percent of the genome was covered, at average read depth of 113× (geometric mean of 103×) for USR1 and 125× (geometric mean of 119×) for USR2 (Fig. S3). Nucleotide mismatches now displayed the

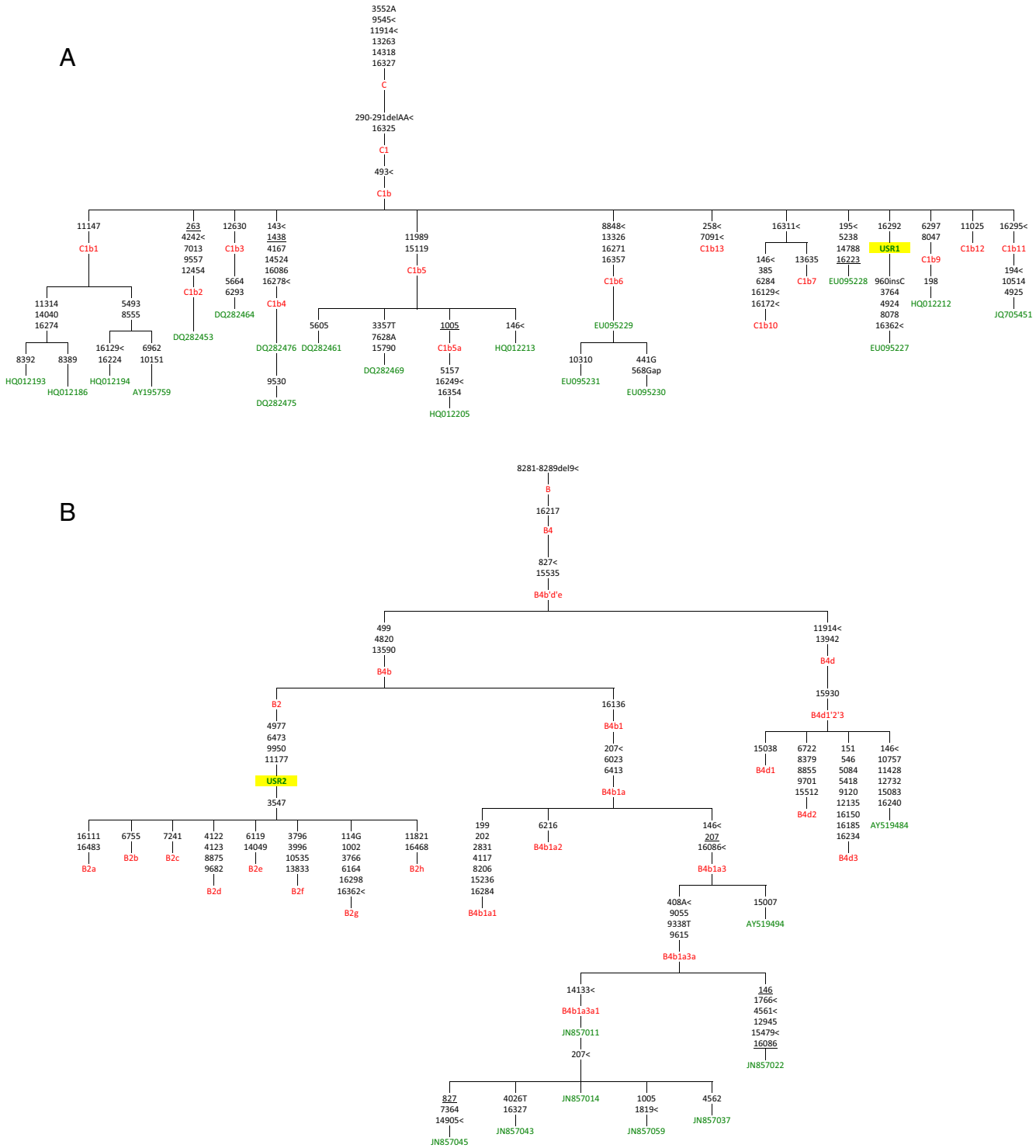

Fig. 2. MP hand-curated phylogenetic trees of (*A*) C1b and (*B*) B4 mtDNA haplotypes. Only a subset of the sequences analyzed in this study are shown, along with the placement of USR1 and USR2. Sequences used in this analysis are listed in green and node assignments are listed in red.

expected damage patterns for degraded samples, although the 5′ read ends still showed some residual unexpected alternative signal (Fig. S4). Although this pipeline lost the necessary flow-space information to make variant calls from Ion Torrent data, visual inspection of the aligned reads confirmed all variants called earlier by TVC. This suggests that the previous quality issue, although masking expected DNA damage patterns at the ends of reads, did not bias the accurate calling of these two samples.

Maximum-likelihood (ML) trees were created from curated alignments of 189 haplogroup C (Fig. 3*A*) and 147 haplogroup B sequences (Fig. 3*B*). USR1 was placed within a large clade shared with C1b, whereas USR2 was placed at the root of known Native American B2 diversity. Both samples exhibit branch length shortening relative to modern Native American sequences, because of their lower number of derived mutations, as expected for aDNA. The best tree by final likelihood score was compared with the results of 1,000 bootstrap runs. Nonparametric bootstrap support on the trees was poor within the Native American specific haplotypes, given the relatively small number of characters providing signal in otherwise highly similar, and polytomous, mtDNA clades (25). Because USR1 and USR2 are contemporaneous, and modern Native American B2 and C1 sequences are observed to have similar coalescence times (6, 12, 26), we investigated the effect of these new sequences on the molecular dates of these clades.

We calculated the coalescence times using an ML-based approach and either a molecular clock corrected for purifying selection (27) or a faster, Bayesian-determined molecular clock based on ancient mitochondrial genomes (28). The C1b clade divergence time was estimated at 16,600 or 13,900 y ago, respectively with the two rates. USR1 was most closely related to an individual of the Arara people of Brazil (EU095227), with an estimated divergence date of 8,200 or 7,000 y ago (a clearly too-recent date given the age of USR1). The Native American-specific B2 clade coalescence time was estimated at 19,100 or 15,900 y ago, respectively (27, 28). All of these dates fall within previously published estimates.

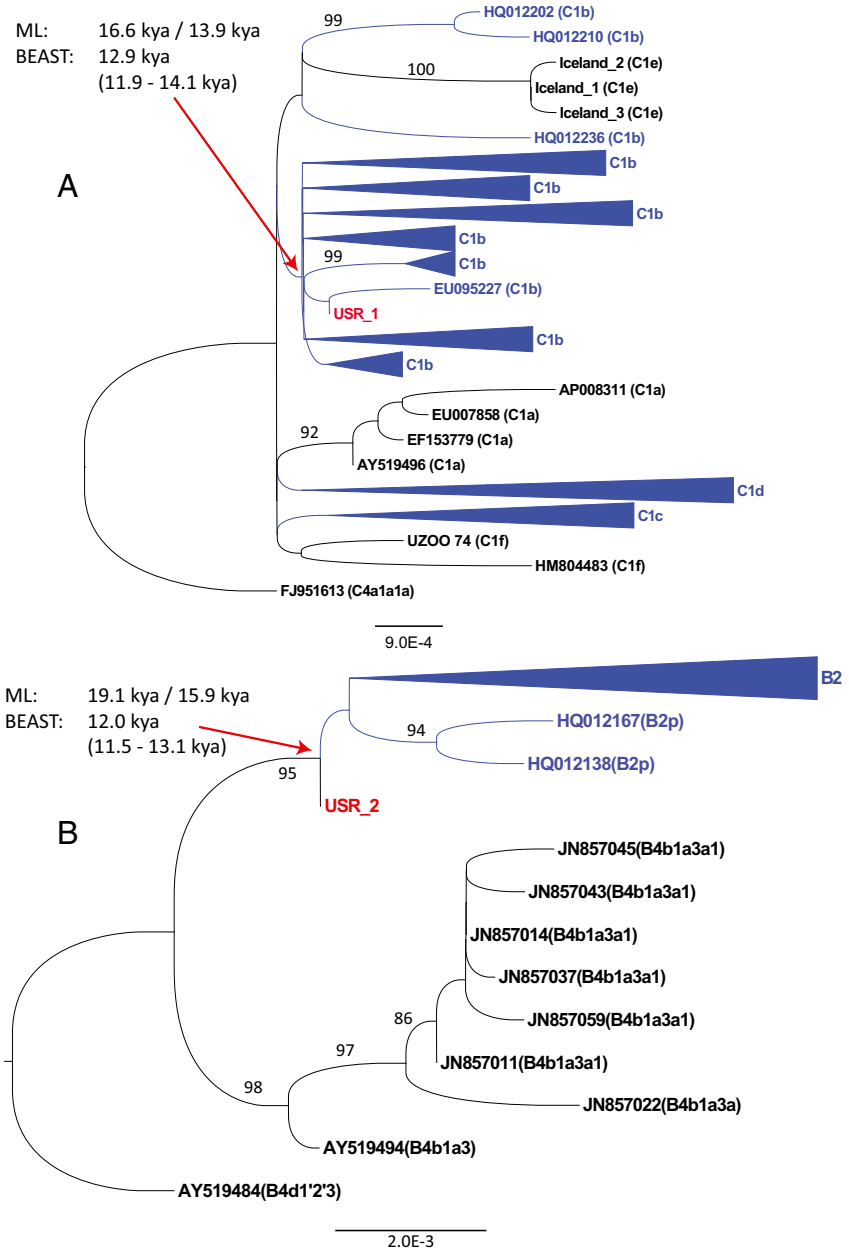

Fig. 3. ML phylogenies of (*A*) haplogroup C1 and (*B*) haplogroup B2. Native American tips and clades are highlighted in blue. USR1 and USR2 are highlighted in red. Some clades have been collapsed for space. Nonparametric bootstrap support for branches are noted for uncollapsed clades with support ≥80%. The ML clade coalescence times using two alternative molecular clocks (27, 28) (see text) or using BEAST 2.2 (29) [mean (95% HPD interval)] are noted for clades C1b and B2. Lower scale bars represent branch length in average nucleotide substitutions per site.

Because the ML-based estimates do not take into account the radiocarbon ages of USR1 and USR2, we used the Bayesian Markov chain-Monte Carlo framework of BEAST 2.2 (29). This Bayesian phylogenetic method uses temporal information from dated sequences to calibrate a molecular clock without relying on geological or paleontological information. Using this approach, we calculated the C1b clade coalescence time at 12,854 y ago (11,853–14,079) [mean; 95% highest posterior density (HPD) interval], with tip dates of 11,500 y ago for USR1 and 8,300 y ago for UZOO-74 (see below). The C1b clade coalescence date is near the younger bounds of the timescales calculated in the literature, although the 95% HPD overlaps with the date previously determined using the faster aDNA-calibrated substitution rate. The B2 clade coalescence time was estimated at 12,024 y ago (11,500–13,085), using a tip date of 11,500 y ago for USR2. This B2 date is also on the later end of previously reported timescales (28). As these estimates are derived from only one (B2) or two (C1) point estimated sequence ages, the analysis can be improved with increased whole-genome sequencing of ancient samples specifically within these clades. The general agreement, however, with the faster Bayesian molecular clock supports relatively young clade coalescence dates.

Discussion

The presence of mtDNA haplotype B2 is somewhat unexpected in this geographic location. This lineage is absent in northern and eastern Siberia (although it is found in the southern periphery) (30), and the pan-American B2 haplotype has not been reported in high-latitude populations of modern indigenous North Americans (Fig. 1). This unusual geographic distribution, coupled with lower restriction fragment length polymorphism haplotype genetic diversity estimates, led to the hypothesis that the B2 lineage was introduced by a later, separate colonization event that did not pass through Beringia. However, following increased sampling and whole mitochondrial genome sequencing, haplogroup B2 phylogenies were shown to have similar star-like phylogenies and coalescence times to the other pan-American founding lineages (12). Moreover, Raff et al. (31) reported two individuals with haplogroup B2 in prehistoric (800 and 490 cal B.P.) populations on the upper Alaska peninsula.

Haplogroup B2 in subarctic interior Alaska at the Upward Sun River site at such an early date suggests it was likely present and polymorphic in the Beringian population that gave rise to the initial dispersal south into the interior of the American continents. Importantly, the finding of haplogroup B2 in far northern interior populations shortly after initial colonization negates the need to postulate models of independent introduction of this mitochondrial lineage through alternative colonization routes. Its absence from modern high-latitude populations now appears consistent with the action of migration and genetic drift in small, dispersed early populations (4) rather than selection or independent introduction. It is noteworthy that haplogroup B was identified at two of the oldest sites in the Americas mentioned earlier: that is, at the ∼8,800 cal B.P. burial at Hourglass Cave in Colorado and in three coprolites dated between 14,270 and 14,000 cal B.P. at Paisley 5 Mile Point Caves in south-central Oregon. Neither site has yielded full mitochondrial genome data.

Haplogroup C is one of the two most common mitochondrial DNA clades throughout northern, eastern, and central Asia (the other being haplogroup D). The wide distribution of haplogroup C suggests it was a component of most migrations in northern Eurasia, with an origin between 30 and 50 kya (32). One daughter clade of the haplogroup is C1, which is composed of an Asian-specific C1a branch previously molecularly dated to 8,500 y ago (32), three Native American-specific (C1b, C1c, C1d) branches previously molecularly dated to 19,000 y ago (12, 26), an Icelandic-specific C1e branch (33), and a novel C1f haplotype sequenced from an individual dated to ∼8,300 cal B.P. (UZOO-74) at the Mesolithic site of Yuzhnyy Oleni Ostrov, North West Russia (25) (although, see Fig. 3*A* for a possibly related sequence, HM804483). Unlike the case for UZOO-74, the USR1 C1b sequence has a clear origin and evolutionary history in the Americas. This result highlights the need for further genomic sequencing of comparably aged C1 lineages: for example, the 10,400 cal B.P. individual from Wizard's Beach, NV and for further sequencing of any C1 lineages in Eurasia.

It is of interest that all five founding macrohaplogroups in Native American populations (A, B, C, D, and X) are represented in the small sample of individuals that lived more than 8,000 y ago in a geographic area stretching from subarctic Alaska to southern Mexico. Four of these macrohaplogroups are found at the three northern North American sites—Paisley Caves, Upward Sun River, and Anzick—dating to over 11,000 y ago. Mitochondrial lineage designation for the majority of the pre-8,000 y ago individuals were determined by low resolution methods of restriction fragment length polymorphism analysis and direct sequencing of PCR products. Only three of the included studies (17–19) used genomic approaches, in addition to the Upward Sun River individuals reported here. Collectively, these results indicate a broad base of mitochondrial diversity in the earliest populations in North America and suggest the importance of postcolonization population dynamics in structuring modern genetic patterns. Cui et al. (13) recently bolstered this inference by reporting four mtDNA genomes from mid-Holocene individuals from coastal British Columbia. The persistence of two unique A2a lineages but the extinction of the D4h3a lineage observed in the transition from ancient to modern Native American populations emphasizes that extant genetic patterns alone can be inadequate indicators of prehistoric population diversity.

Although the Upward Sun River population postdates the end of the original dispersal of populations into North and South America by a few thousand years, it is temporally and geographically the closest known to the larger interior Beringian population that was the source of that earlier migration. Furthermore, if the Beringian population was subdivided in refugia, as recently suggested (4), the geographic structure seen in modern indigenous North Americans may reflect early population differentiation and multiple dispersals of small, isolated groups in interior Beringia to interior North America. Available archaeological and genetic data from Late Pleistocene contexts in North America are consistent with the origin of Native American mitochondrial genomes in populations resident in interior Beringia with subsequent dispersal southward sometime before 14–16 kya. The distribution of founding mitochondrial lineages in ancient samples of the Americas suggests an early movement of interior Beringian peoples southward at colonization, followed shortly by similar dispersal along the Pacific coast. The ancient mitochondrial genomes of the two contemporaneous Upward Sun River infant burials provide an important anchor between modern patterns of genetic variation and the inferences that may be drawn from retrospective population genetic analyses.

Conclusion

The genomic results on the Upward Sun River infants are significant for several reasons. First, they not only double the number of late Pleistocene burials that have been characterized genetically, but they are also the only example to date of multiple burials from a single North American Pleistocene-aged archaeological site. Second, the genomic results from the USR infants support the Beringian route into the Americas and imply substantial interior Beringian genetic variation in the Late Pleistocene, consistent with expectations of the Beringian Standstill Model. Phylogenetic coalescent dates informed by the sample radiocarbon ages suggest more recent expansions for the Native American C1 and B2 clades than has previously been suggested. Third, these results clarify the infants' biological relationship to one another, something that morphological data could not do (23). Fourth, the fact that the infants are contemporaneous in time and buried together in a single act speaks to population diversity in ways that single sample reports cannot. And fifth, the dual burial of maternally unrelated infants (although perhaps paternally related), suggests additional hypotheses regarding mortuary practices and social and ceremonial behaviors present at this early time; this line of investigation may be addressed in the future by both nuclear genomic analyses of the infants, as well as continued elaboration of the archaeological context of the site.

Materials and Methods

USR1 and USR2 were complete and located 8–10 cm apart at the bottom of the pit feature at the Upward Sun River site, located in the middle Tanana River valley. Two petrous specimens were selected for DNA analyses given their overall mass and high density. Details on site formation, chronology, site disturbance, and excavation protocols are reported elsewhere (21–23, 34). Destructive analysis and genetic sequencing of the material was formally allowed by a Memorandum of Agreement with all interested parties. DNA was extracted using a silica-based method and initially amplified using established protocols. Extracts were prepared into Ion Plus Fragment libraries (Life Technologies) with no DNA fragmentation or size selection. Fragments were blunt-end ligated with adapters containing laboratory-specific custom barcodes. Mitochondrial DNA was captured by hybridization (24) and each sample library was sequenced on its own Ion PIv2 chip (Life Technologies). Read processing was completed either within Torrent Suite, with variants called using TVC, or reads were processed using offline tools to determine DNA damage patterns. Haplotypes of consensus mitochondrial genomes from these variants were identified by MP and phylogenetic trees of all known related sequences were created by ML. Coalescence dates for the clades within these trees were calculated using ML-based or Bayesian-based phylogenetic methods. Work was performed in a dedicated aDNA facility using established clean room protocols. Blanks were included at all steps in the process before sequencing and no laboratory personnel carry the haplotypes reported here. An expanded discussion of detailed materials and methods can be found in *SI Materials and Methods*, Figs. S1–S6, Tables S1–S3, and Datasets S1 and S2.

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