

ANTHROPOLOGY

Ancient genomes from Ladakh reveal 2800-year-old admixture between Tibetans and South Asians

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Reconstructing population history is harder in South Asia than in many other world regions due to a paucity of ancient DNA. We report genome-wide data for 10 individuals from Old Lady Spider Cave, which lies 4000 meters above sea level in the Himalayan region of Ladakh and dates to around 1500 years before the present (B.P.). These individuals were genetically homogeneous and had an ancestry signature rare in South Asians today: admixed in roughly 50–50% proportions between a population well proxied by present-day North Indians and another genetically similar to ancient Tibetans. By analyzing the typical sizes of segments of DNA inherited from each of these ancestral populations, we find that admixture of these groups began at least 50 generations before the date of the individuals, that is, by around 2800 B.P.

INTRODUCTION

The Indian Union Territory of Ladakh lies at the crossroads of ancient trade routes connecting the Tibetan Plateau, parts of Central Asia including the Tarim Basin and the Hindukush-Pamir corridor, Kashmir, and South Asia. Petroglyphs dating to the Bronze Age [5000 to 3000 before the present (B.P.)] document cultural links with Central Asian cultures, such as the Okunevo and Afanasievo of the Altai and south Siberia, and the Andronovo of west Central Asia (1–5). Petroglyphs dating to the Iron Age (3000 to 2000 B.P.) in the “Animal” or “Steppic Style” provide links with the Saka cultures of the west Central Asian steppes (1, 3, 5).

After 2300 B.P., an east-west divide appears in the material culture record (6, 7). The east of Ladakh is characterized by “Cord Ware” style ceramics (not to be confused with the 5000 to 4400 B.P. European culture of the same name) found in the wider West-Tibetan and West-Himalayan world, including in Spiti, Guge, Purang, and Mustang. The west of Ladakh, where Old Lady Spider Cave is located, is marked by Central Asian-influenced ceramics with red slips and distinctive shapes and decoration, occurring in Hellenistic (2300 to 2000 B.P.), Kushan (2000 to 1600 B.P.), and post-Kushan (1600 to 1100 B.P.) times (5). There are also Kharoṣṭī and Brāhmī inscriptions, typical of the Kushan and post-Kushan world (8). According to oral traditions, Ladakh was once inhabited by speakers of language groups including Dardic (Indo-European speakers found today across northwest India, northern Pakistan, and northeast Afghanistan), Mon (across the Himalayan arch from India to Burma), Turkic, Mongol, and Tibetic. A limitation is that we do not know when any of the speakers of these languages first came to Ladakh, and these ethnonyms must reflect only a subset of the groups in the region in prehistoric times.

The Old Lady Spider Cave, or Abi Srinjamo Bao, is located at an altitude of 4000 m in the pastureland above the village of Yogma Kharbu and is one of the few deep caves of the region (Fig. 1). A first large room contains petroglyphs and remains of looted cist graves. A long and narrow tunnel leads to a second large room, where bones are densely scattered in two main clusters. A third room can be accessed from the second room, and its ceiling is only 50 cm high. Bones are also scattered in this room, although more sparsely. On the basis of highly preserved skeletal elements like hand and foot bones, it is likely that the bodies were kept on the cave floor and not moved after their primary deposition (9). As the cave has been visited by many people for centuries, however, the bones are disturbed. One of the main aims of the excavation was to verify the context of the bones and to determine whether bones were present below the existing floor of the cave (9).

Excavations were conducted in 2021 by a group directed by two of the authors (V.M.-T. and Q.D.), under the auspices of the Archaeological Survey of India (ASI). The collection represents all age groups (infants to elders) and both adult males and females (9). Other findings include bones with naturally mummified tissues (skin, ligaments, and muscles), including two mummified hands. With the goal of minimizing contamination, we wrapped bones and mummified tissues in clean aluminum foil and transferred them to clean plastic bags at the Deccan College Post-Graduate and Research Institute (Pune). We obtained permission from the ASI to export the samples to Harvard University to study them using specialized ancient DNA preparation and in-solution enrichment procedures. V.M.-T. hand-carried the samples to Boston, United States for this analysis.

Dataset assembly

We extracted DNA from 11 specimens using protocols adapted to the specific type of sample (bone or soft tissue). The samples were eight bones (a clavicle, a femur, a fibula, a humerus, a radius, a tarsal, an ulna, and a rib) and three pieces of soft tissue (all from the pelvic area) (10–13). We generated 52 double-stranded (14) and 12 single-stranded (15) sequencing libraries, enriched them in solution using the “Twist Ancient DNA” assay targeting 1,352,535 single-nucleotide polymorphisms (SNPs) (16), and sequenced them on Illumina NovaSeq instruments aiming for 20 to 30 million paired sequences of 101 base pairs each for each library (table S1). We aligned the sequences to

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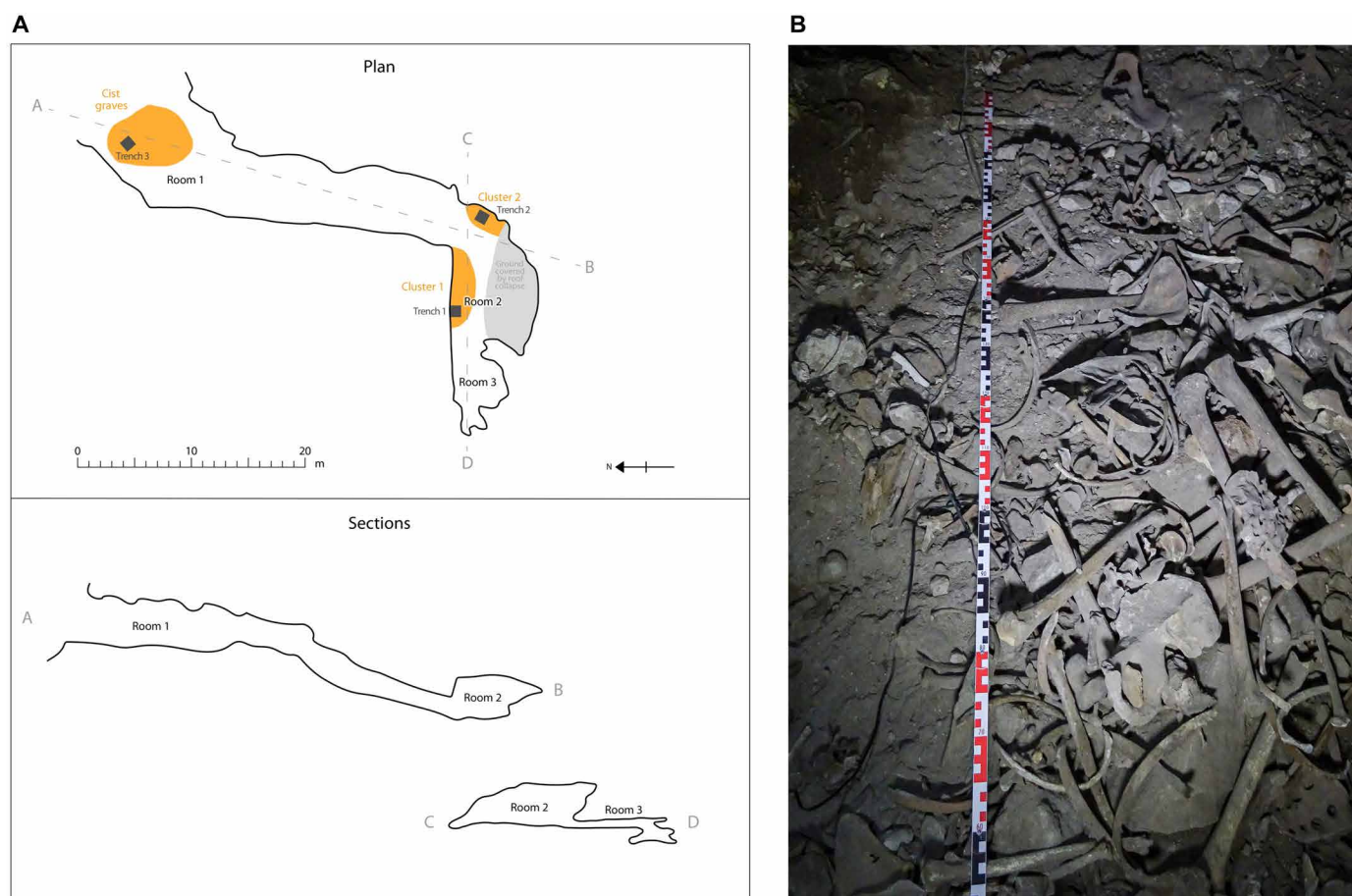


Fig. 1. The Old Lady Spider Cave. (A) Plan and section of the cave, drawn from a previously unpublished point cloud shared with permission by D. Rosati. Orange shading shows where human remains were found, while gray shading shows a part of the cave covered by a roof collapse. (B) Previously unpublished photograph of bones in room 2, taken by the authors during the excavation.

the human reference genome and carried out almost all of our analyses at a set of 584,131 SNPs on chromosomes 1 to 22 that had also been genotyped in hundreds of relevant modern populations using the Affymetrix Human Origins SNP genotyping array (17).

After quality control and merging data obtained from two samples that we determined on the basis of the genetic results to be from the same individual (S36588 and S36589, referred to in Table 1 as I36588), we had data from 10 unique individuals. Six represented by bones yielded excellent data with 0.85- to 12.5-fold average sequence coverage on targeted positions (median 6.3-fold). One individual represented by a pelvic tissue sample had usable but not particularly high-quality data (0.048-fold coverage). The remaining three individuals represented by two pelvic tissue samples and one clavicle had too little data for genome-wide analysis (0.0016- to 0.015-fold) (Table 1).

We determined genetic sex based on the ratio of Y to X chromosome sequences, identifying six genetic females and four genetic males (all three tissue samples were from males). On the basis of the ratio of the number of allelic differences between two sequences of the same individual, versus two sequences of different individuals (18), we determined that I36572 is a second degree relative of I36567 and a second- to third-degree relative of I36568.

We generated accelerator mass spectrometry radiocarbon dates on the eight bone samples (table S2). The 95% confidence intervals

for the dates overlap in the range of 1513 to 1422 calibrated years before the present (cal yr B.P.) (16). The union of 95% confidence intervals is 1690 to 1347 cal yr B.P.

Ancestry intermediate between North Indians and Tibetans

We carried out principal components analysis (PCA) using smartpca (version 18720, run with the option `roundmode:YES`) (19). We co-analyzed genome-wide data from French, Han Chinese, several Tibetan plateau populations [modern Sherpa, modern Tibetans, and 4600-year-old Zongri (20, 21)], and a variety of populations of the “Indian Cline,” referring to a previously described genetic gradient of present-day South Asians with relatively more (Pathan and North Indian Brahmins) and less (Palliyar) genetic sharing with West Eurasians (22, 23). We then projected the Old Lady Spider Cave individuals, who we call Old Ladakh in what follows, and relevant modern populations including Minero from Ladakh (24), Balti from Ladakh (25), and more than a thousand other individuals from diverse Indian populations that make up the Indian Cline including Dogra from Jammu and Kashmir (table S3 gives the coordinates of each plotted point as well as their population affiliations) (22, 23). The first eigenvector separates people with primarily East Asian ancestry, and the second separates groups of the Indian Cline (Fig. 1). A homogeneous cluster of the seven Old Ladakh individuals with

highest-quality data, as well as the Minero and Balti populations from the Himalayan region, falls along a line joining Tibetans to a point on the Indian Cline that includes both Dogra and North Indian Brahmins. This is the pattern expected if their ancestry is admixed between groups related to these populations. Except Balti and Minero, we are not aware of other modern groups plotting in a position similar to Old Ladakh, who have a unique ancestry profile for South Asia (Fig. 2).

No fitting two-source model for Old Ladakh

To quantify the mixture proportions and distinguish models, we used qpAdm, part of the ADMIXTOOLS software package (version 2201, run with options “oracle:NO” and “allsnps:YES”) (17). qpAdm allows us to propose a model for the pool of Old Ladakh individuals as a mixture of populations that can be proxied by a set of “left source”

populations hypothesized to be descended without mixture from the same origin as the true source populations. qpAdm also provides a formal statistical test of fit of the model to data. For our analyses, we often use modern populations as proxies for the ancient ones; as an example, motivated by the PCA, we used North Indian Brahmins as a source in our modeling of Old Ladakh, although we do not think that Indians admixing into Ladakh were culturally “Brahmins” and identifying these ancient people with modern cultural groups makes little sense. Using modern proxies as a source in qpAdm is well motivated from a statistical point of view, however, and is a case for which qpAdm has a clear interpretation: The methodology was developed a decade ago for the application of studying European population history at a time when ancient DNA from that region was scarce as remains the case for South Asia today (26). If qpAdm produces a passing *P* value, the proposed proxies for the sources are

Table 1. Summary of genetic results. Note that we computed mean coverage on a core set of SNPs on chromosomes 1 to 22. We restricted population genetic analyses to a maximum of 584,131 SNPs that had also been genotyped in large numbers of modern samples. The number of libraries is given in parentheses in case not all pass the quality control. Full details of the libraries used to produce these merged data are provided in table S1. mtDNA, mitochondrial DNA.

Genetic ID	Skeletal code	Sample type	Mean coverage	SNPs covered at least once	Libraries	Genetic sex	Y chrom	mtDNA
I36568	21 (S9)/23 (S10)	Rib/radius	12.5	583,069	8	F	..	U5a1d1
I36572	33 (S15)	Femur	7.7	581,461	4	F	..	U5a1d1
I36567	17 (S7)	Humerus	7.5	580,053	4	F	..	M65a + @16311
I36570	25 (S11)	Tarsal	5.1	554,566	2	M	R2b1	U2b1
I36565	4c	Ulna	1.57	457,862	4	F	..	F2g
I36566	15 (S6)	Fibula	0.85	342,683	4	F	..	M4"67
I36701	11	Pelvic tissue	0.048	27,916	5 (of 12)	M	J1	U7a3b
I36571	31 (S14)	Clavicle	0.015	3,512	2	F	..	D4j1b
I36700	9	Pelvic tissue	0.0084	4,453	4 (of 12)	M	..	R
I36697	5	Pelvic tissue	0.0016	690	1 (of 12)	M	..	..

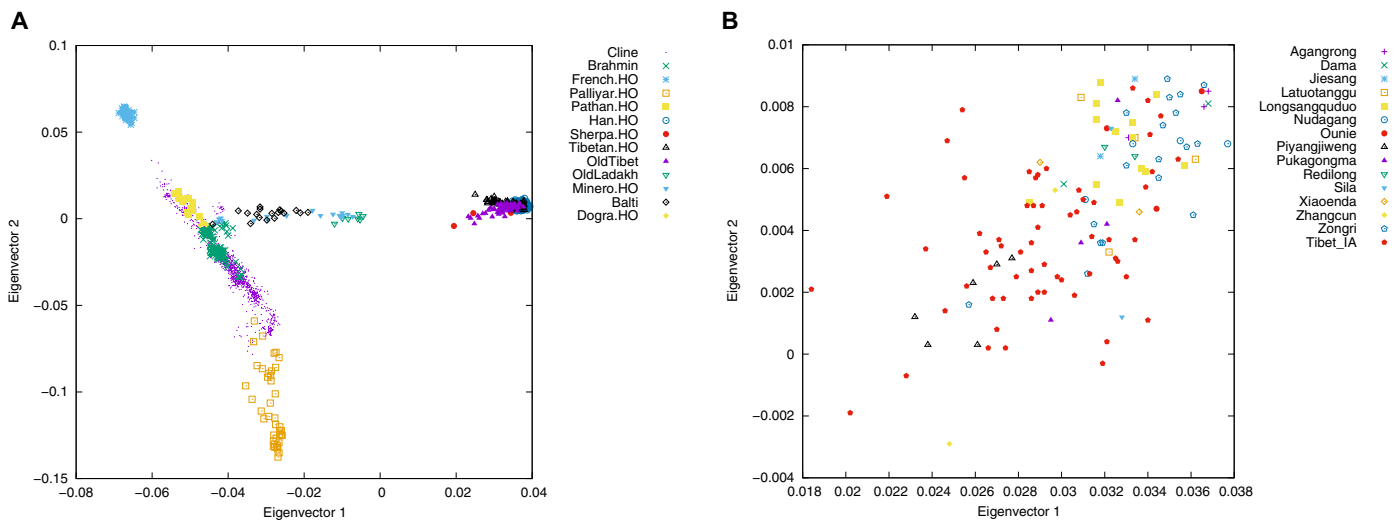


Fig. 2. PCA reveals the relationship of Old Ladakh individuals to selected ancient and modern individuals. (A) We define the axes of the PCA using selected Tibetan individuals from before the Iron Age (“OldTibet”), modern European and East Asian populations, and a variety of groups on the Indian Cline including Pathan (extreme northern end of the Cline), several North Brahmin groups, and Palliyar (at the extreme southern end). We project the seven higher coverage Old Ladakh individuals, as well as Dogra, Minero, Balti, and 1153 individuals from diverse present-day Indian populations (tiny purple dots). (B) Blow-up showing the weak structure within Old Tibet individuals.

consistent with descending without mixture from the same groups as the true source populations. Even if the proxies postdate the true sources, we can use them to obtain unbiased estimates of mixture with valid SEs.

To provide statistical leverage to falsify proposed models, qpAdm requires a set of “right outgroup” populations related differentially to the modeled population and the left sources. For this application, we used eight right outgroups (table S4). Four are modern groups genotyped on the Affymetrix Human Origins (HO) SNP array: 61 “French.HO” individuals to represent Europeans (17, 27, 28); 107 “Han.HO” individuals to represent East Asians (17, 29); 41 “Palliyar.HO” individuals to represent a South Asian group on the Indian Cline with minimal West Eurasian relatedness (23); and 26 “Juang.HO” individuals to represent a South Asian Austroasiatic-speaking group with even less West Eurasian relatedness (23). Four right outgroups are ancient: a pool of 10 diverse ancient Africans with little or no West Eurasian ancestry and dating to 8000 to 1000 B.P. “OldAfrica” (30–33); 9 pastoralists from the western Zagros mountains site of Ganj Dareh dating to around 10,000 B.P. “Iran_GanjDareh_N.AG” (28, 34, 35); 21 hunter-gatherers dating to around 7000 B.P. from the middle to lower Volga region of Russia from the Ekaterinovka Mys site “Russia_Ekaterinovka_Eneolithic.AG” (35); and 3 Mesolithic hunter-gatherers from the Amur River region of far northeastern China dating to around 11,000 B.P. “China_AmurRiver_Mesolithic.AG” (36).

We performed an initial analysis in which we tested 220 possible two-way mixture models for Old Ladakh, motivated by the position of these individuals in the PCA. These models included as proxy for the ancient South Asian–related source 1 of the 4 North Brahmin groups, and, as a proxy for the Tibetan-related source, 1 of 55 modern or ancient East Asians groups (table S4).

Table 2 shows all two source models for Old Ladakh, yielding a *P* value greater than 0.001 (table S5 gives results for all models tested). All include one source related to Brahmins in North India contributing about 50% ancestry and a second related to ancient Tibetans. The exception is that in the four best-fitting models, the non–South Asian source is an individual from the Amur River region of far

northeastern China dating to ~1650 B.P. (37). That we only observe good fits (*P* > 0.05) using this Amur River individual should not be interpreted as evidence that the true source is from this region, since with only around 40,000 SNPs and a single individual, there is limited power to reject incorrect models. Since geographically proximate Tibetan populations fit second-best but do not fit exactly, we hypothesized that the true source is a Tibetan group that is not perfectly proxied by any populations we had available, perhaps one with more Amur River relatedness. The degree of Amur River relatedness varied in ancient Tibetans (Fig. 2B), and we observe direct evidence for this via the statistic *f*₄(OldAfrica, China_Amur-River_Mesolithic; Agangrong, Zhangcun), which is significantly nonzero for the two ancient Tibetan groups Agangrong and Zhangcun (*Z* = −6.4). Similarly, previous work has shown that Tibetan groups varied in their degree of relatedness to ancient people from Xinjiang like Bronze Age Xiaohu (20). On the basis of these considerations, the most plausible explanation for our observations is that the true Tibetan source population had a slightly different mixture of ancestries than any of the ancient Tibetans for which we had data but fell within their sampled variation. We find statistical evidence that Tibet itself likely contained the correct northern ancestry source—from an unsampled population with an ancestry intermediate between the ancient groups for which had data—by attempting to fit Old Ladakh individuals using qpAdm with three sources: one Indian and two Old Tibetan (Amur River basin or Xiaohu individuals were not used as sources in this analysis). We identify several models that yield *P* values > 0.05, although we do not discuss them because the SEs on the admixture coefficients of the Tibetan populations are extremely large as the populations are so close genetically, and hence, the fitted coefficient values are meaningless.

Old Ladakh people had roughly half their ancestry from an unsampled population genetically similar to but not exactly the same as sampled ancient Tibetans

To identify fitting models for the ancestry of the Old Ladakh individuals, we tested 4940 three-source qpAdm models with three sources: (i) 1 of 260 populations from South Asia, (ii) 1 of 19 Ancient Tibetan

Table 2. Best two-way mixture models for Old Ladakh. Full results in table S5.				
South Asian–related population	Tibet/China-related population	South Asian related	SE	<i>P</i> value
Brahmin_Haryana.HO (23)	China_AR_IA.SG (37)	46.8%	2.1%	0.305
Brahmin_Nepal.HO (23)	China_AR_IA.SG (37)	47.4%	2.0%	0.235
Brahmin_Uttarakhand.HO (23)	China_AR_IA.SG (37)	51.0%	2.2%	0.169
Brahmin_UP.HO (23)	China_AR_IA.SG (37)	47.7%	2.1%	0.094
Brahmin_Nepal.HO (23)	TibetanPlateau_Long-sangquduo.AG (20)	52.2%	0.8%	0.040
Brahmin_Haryana.HO (23)	TibetanPlateau_Long-sangquduo.AG (20)	51.1%	0.9%	0.019
Brahmin_Haryana.HO (23)	Zongri (20)	52.7%	0.9%	0.008
Brahmin_Haryana.HO (23)	TibetanPlateau_Latuo-tanggu.AG (20)	51.2%	0.9%	0.007
Brahmin_Nepal.HO (23)	TibetanPlateau_Latuo-tanggu.AG (20)	52.4%	0.9%	0.006
Brahmin_Nepal.HO (23)	Zongri (20)	53.9%	0.8%	0.003

populations, and (iii) people of the genetically distinctive Xiaohe culture from Bronze Age Xinjiang approximately 5000 to 4800 B.P. (China_Xinjiang_Xiaohe_BA.AG) (table S6) (38). For these analyses, we modified the right outgroup set used for two-source qpAdm models. We removed Palliyar.HO as Juang contains a great deal of Ancestral South Indian ancestry and Palliyar added no information (and reduces the sensitivity of the analysis). We also added three right outgroups that we found after exploration to provide additional leverage to distinguish between East Asian–related ancestry. These were a pool of four Late Paleolithic hunter-gatherers from the Amur River region of far northeastern China dating to 15,000 B.P. “China_AmurRiver_LPaleolithic.AG” (36); the low coverage Amur River region Iron Age individual dating to around 1800 B.P. who was part of the only fitting models in the two-way qpAdm analysis (37); and a pool of eight individuals from the Xiongnu culture dating to around 2000 B.P. ‘Russia_Buryatia_Xiongnu.AG’ (39).

We identified 80 three-way models fitting at $P > 0.05$ that inferred mixture proportions for all three sources between 0 and 100% (results for all models including SEs are in table S7). Restricting to groups represented in more than one fitting model, all included a North Indian–related source (typically 46 to 50%), a source related to one of the populations we sampled from ancient Tibet (43 to 52%), and a Xiaohe-related source which is likely to capture additional unsampled ancestry from Tibet (Xiaohe is too old to be the true source, but variation in Xiaohe-relatedness allows us to capture ancestry variation in ancient Tibet) (5 to 8%) (Table 3). The exceptions were Bahun.HO from Nepal (3 model fits) and Minero.HO from Ladakh (11 models fits), whose estimated contributions to fitting models were higher than for other South Asians at 59 to 65%, reflecting the fact that they themselves are mixes of typically Indian Cline and East Asian ancestry (22, 26, 40). We conclude that since the vast majority of South Asian populations yield mixing coefficient of 46 to 50% with small SEs of around 1% (Table 3), the mixture of South Asians and Tibetans is close to half-half, consistent with their positioning in the PCA of Fig. 2A.

To probe the robustness of our best-fitting models, we focused on one that included North Indian Brahmins from Uttarakhand, ancient Tibetans from Latuotangu, and ancient Xiaohe ($P = 0.17$; table S7). We then used the same eight right outgroups as for our primary qpAdm analysis and carried out 492 qpAdm runs in turn, adding diverse populations to the right. We observe some poor fits (table S8) when adding to the right modern Himalayan populations such as Burusho.HO, Kalash.HO, and Minero.HO. This implies genetic drift shared between these groups and the Old Ladakh individuals, as expected for flow of Old Ladakh–related people into the ancestors of these groups. Thus, the Old Ladakh population did not completely disappear and instead left a legacy in present-day people.

It is tempting to interpret the inclusion of 5 to 8% Xiaohe ancestry in our fitting models as evidence that this isolated Bronze Age group contributed ancestry to Old Ladakh people. However, this interpretation is not warranted and is brought into question by our analyses. When we move to the right of our qpAdm model Shamanka Eneolithic from Russia or Khovsgol Late Bronze Age from Mongolia, the models fail (table S7), as would be expected for these groups having received gene flow from a Xinjiang- or Amur River–related source that lived later than the very ancient and isolated Xiaohe. The conservative interpretation of our results is that we do not have access to data from exactly the right Tibetan-related population but that the true Tibetan-associated source population had a different mixture of ancestry ingredients present in Tibetan-related populations (Tibetan

associated, Xinjiang associated, and Amur River associated) compared to the specific individuals for which we have ancient DNA.

Together, our data are consistent with Old Ladakh individuals being a mixture of approximately half ancestry related to North Brahmins and Dogra and half ancestry related to sampled ancient Tibetans but not perfectly proxied by any available Tibetans in our dataset. These results are also compatible with the patterns observed at uniparentally transmitted mitochondrial DNA sequences and Y chromosomes (Table 1). Six haplogroups are typically South Asian and nearly absent in Tibetans: both the Y haplogroups R2b1 and J1 and four mitochondrial haplogroups M65a + @16311, U2b1, and M4’67 U7a3b (<https://yfull.com/mtree> and <https://yfull.com/tree/>). Two mitochondrial haplogroups are common in Tibetans and nearly absent in South Asians: F2g and D4j1b. One mitochondrial haplogroup, R, is consistent with either a South Asian or a Tibetan source. Last, a pair of second- to third-degree relatives carry the mitochondrial haplogroup U5a1d1, characteristic of modern and ancient North Europeans including from the Caucasus and Lower Volga area, but both are rare in South Asia today. The most plausible hypothesis is that this haplogroup arrived in North India as a low-frequency allele carried by people with steppe ancestry.

Old Ladakh ancestors formed through mixture by around 2800 B.P.

To estimate when the mixture of South Asian- and Tibetan-related ancestry occurred in the ancestry of seven Old Ladakh individuals with highest coverage data, we ran the software DATES version 4555 (41, 42). We used a pool of 35 North Indian Brahmin individuals from four areas (Haryana, Nepal, Tiwari, Uttarakhand, and Uttar Pradesh) as a proxy source to represent the ancient South Asian–related source and 143 modern people with Tibetan-related ancestry as a proxy source to represent the ancient Tibetan-related source. DATES reveals a clear covariance of ancestry with distance (Fig. 3). The data are well fit by a single exponential function, implying that the population was insulated from later waves of mixture. Assuming 29 years per generation, we infer the admixture occurred 1343 ± 140 years before the date of the individuals or roughly 2800 B.P. Previous work showed that Iron Age people from what is now northern Pakistan had all three of the deep ancestry components that contributed to the modern Indian Cline by this time, including Steppe-related, Iran-related, and indigenous Ancient South Indian–related (41). However, those individuals did not have these ancestries in the right proportions to be on the Indian Cline. The ancestry of the Old Ladakh individuals suggests the people with ancestry at the location of the Indian Cline where present-day Dogra and North Indian Brahmins fall already existed by ~2800 B.P.

Old Ladakh people carried a variant from Denisovans conferring high-altitude adaptation

Modern and ancient Tibetans frequently carry an allele that confers adaptation to a high-altitude environment, which introgressed from archaic Denisovans (43). To test for the presence of the Denisovan haplotype carrying this allele in the newly reported individuals, we used the raw data from the individuals we sequenced to identify 11 SNPs that are diagnostic for the Denisovan haplotype and are effectively ascertained after our enrichment process. There are reads covering at least 1 of these 11 SNPs in five individuals (table S9). For four individuals, there are enough reads to make a confident diploid genotype determination (zero, one, or two copies of the Denisovan

Table 3. Fitting three-way mixture models for Old Ladakh. We tabulate all 80 models that fit at $P > 0.05$ and have mixture proportions of 0 to 1. The left lists all South Asian sources (of 260) yielding at least one passing model, and the right lists Tibetan sources (of 19). We show the average of inferred mixture proportions over all individual passing models; SEs for all three sources in individually passing models are typically 0.01 and are given in table S7. Full details for all 4940 tested models are also given in table S7. qpAdm is run with the option allsnps: YES; we obtained similar results with allsnps: NO.

South Asian source yielding fits (260 tested)	Models fitting at $P > 0.05$ (of 19 tested)	South Asian source proportion	Mean Tibetan source proportion	Mean China_Xinjiang_Xiaohe_BA.AG	Tibetan source yielding fits (19 tested)	Models fitting at $P > 0.05$ (of 260 tested)	Mean South Asian source proportion	Mean Tibetan source proportion	Mean China_Xinjiang_Xiaohe_BA.AG
Gujjar.HO	1	0.41	0.50	0.08	Tibetan.DG	6	0.43	0.50	0.08
Khatri.HO	3	0.44	0.51	0.06	Latuotanggu	18	0.48	0.46	0.05
Sindhi_Pakistan.HO	2	0.44	0.50	0.06	Dama	12	0.49	0.46	0.06
Lohana.HO	1	0.45	0.50	0.05	Ounie	16	0.49	0.46	0.05
Pandit.HO	4	0.46	0.48	0.06	Zhangcun	2	0.50	0.44	0.06
GujaratiA.HO	5	0.46	0.49	0.05	Agangrong	19	0.52	0.44	0.05
Yadav_Rajasthan.HO	5	0.46	0.49	0.05	Long-sangquduo	2	0.57	0.39	0.04
Yadav_UP.HO	3	0.47	0.48	0.06	Tibet_IA	1	0.63	0.33	0.04
Dogra.HO	5	0.47	0.47	0.06	Sila	1	0.63	0.31	0.06
Brahmin_Haryana.HO	5	0.47	0.48	0.05	Jiesang	1	0.65	0.30	0.05
Brahmin_UP.HO	2	0.47	0.48	0.05	Nudagang	1	0.66	0.29	0.05
Rajput.HO	4	0.47	0.47	0.06	Zongri	1	0.67	0.29	0.04
Muslim_Kashmiri.HO	4	0.48	0.47	0.06					
Sikh_Jatt.HO	2	0.48	0.47	0.05					
GujaratiB.HO	2	0.48	0.47	0.06					
Bhumihar_Bihar.HO	4	0.48	0.47	0.05					
Kshatriya_Durgvanshi.HO	2	0.49	0.46	0.05					
Brahmin_Nepal.HO	5	0.50	0.46	0.04					
Brahmin_Tiwari.HO	1	0.50	0.46	0.05					
Bhumihar_UP.HO	1	0.50	0.45	0.05					
Brahmin_Uttarakhand.HO*	4	0.50	0.44	0.06					
Bahun.HO*	3	0.59	0.35	0.06					
Bink.HO*	1	0.60	0.34	0.07					
Minero.HO*	11	0.65	0.30	0.05					

*Detected as having East Asian-related mixture so the South Asian ancestry proportions are likely overestimates.

haplotype); for the fifth, we can only infer a single chromosome, giving calls on nine chromosomes altogether. Of these, one carries the Denisovan haplotype. Approximately half of the Ancient Tibetan chromosomes have this haplotype, and we estimate that Ancient Tibetans contributed about half the ancestry of the Old Ladakh individuals. Thus, in the absence of natural selection, about a quarter of chromosomes of Old Ladakh are expected to have the Denisovan haplotype, which gives an expected number as 2.25. Seeing only 1 is

not statistically unexpected. All we can say is that after admixture, this haplotype was not under strong positive selection.

DISCUSSION
The ancient individuals we analyzed from Ladakh are of great interest. First, there is little genetic data available from anywhere in the Indian subcontinent that is as old as these individuals. Second, their

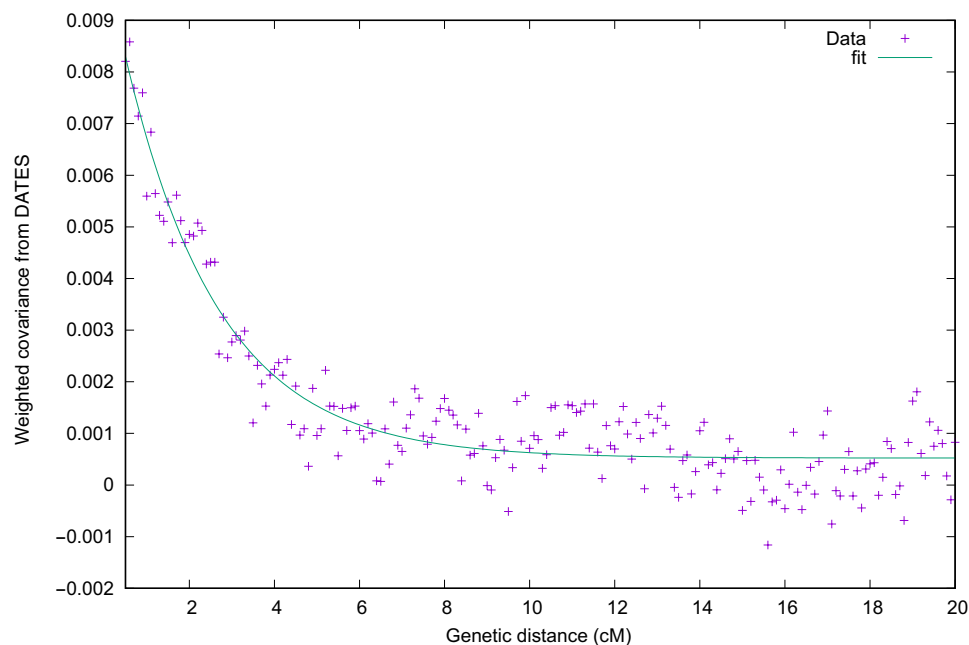


Fig. 3. Correlation of ancestry with genetic distance in the Old Ladakh individuals. We observe a decay of admixture linkage disequilibrium on the scale of 2 centimorgans (cM), implying that admixture began at least 50 generations before they lived. The y axis is the covariance of which admixing population contributes a target allele. Theory shows that this quantity decays exponentially as a function of genetic distance on the x axis, with the decay rate proportional to the number of generations since admixture (41, 42).

ancestry is unusual: Very few groups today have a similar ~50–50% South Asian/Tibetan mix. Third, we are likely learning about the deep history of Ladakh, although a caveat is that the very old admixture did not necessarily occur in Ladakh itself. For example, admixture could have occurred in what is now Nepal, and then members of the admixed population moved to Ladakh. Fourth, the data provide direct evidence of contacts between Ladakh and Tibet a century before the rise of the Tibetan Empire (44).

Our results do not imply that the detected admixture took place in Ladakh. However, we have shown that the people from Old Lady Spider Cave were part of a population that was long-established—arising through admixture at least around 1300 years before the time the sample people lived—and likely made a contribution to people living today in the Himalayan region including Ladakh. In particular, groups like the Minero from Ladakh have qpAdm-based evidence of gene flow from an Old Ladakh group (table S8), and they and Balti fall on a gradient of differential relatedness between North Indian groups and Old Ladakh (Fig. 2). In particular, when we carried out a qpAdm analysis with right outgroups Old Africa, China_AR-IA.SG, Han.HO, Juang.HO, Brahmin_Haryana.HO, and Tibet_IA, Minero fit well ($P = 0.24$) as a mixture of Dogra ($44 \pm 1\%$) and Old Ladakh ($56 \pm 1\%$), and Balti fit marginally ($P = 0.04$) as a mixture of Dogra ($55 \pm 1\%$) and Old Ladakh ($45 \pm 1\%$). An important direction for future work is to sample additional remains to understand the geographic and temporal span, as well as the cultural associations, of this previously unknown population that lived in the Himalayan region for more than a millennium.

MATERIALS AND METHODS

Provenance

Excavation of the human remains was carried out in 2021 by a team led by two of the corresponding authors (V.M.-T. and Q.T.), under

the auspices of the ASI, which gave permission for this work (9). Permission for sending the archaeological materials to Harvard University for specialized ancient DNA analysis and radiocarbon dating was obtained from the ASI in a letter dated 30 December 2021, and they were hand-carried by corresponding author V.M.-T. Open science principles require making all data used to support the conclusions of a study maximally available, and we support these principles here by making fully publicly available not only the digital copies of molecules (the uploaded sequences) but also the molecular copies (the ancient DNA libraries themselves, which constitute molecular data storage and reside at Harvard Medical School). Those researchers who wish to carry out deeper sequencing of libraries published in this study should make a request to corresponding author D.R. We commit to granting reasonable requests as long as the libraries remain preserved in our laboratories, with no requirement that we be included as collaborators or coauthors on any resulting publications. The remaining skeletal samples are housed at Deccan College, and we commit to granting reasonable requests to study them from an anthropological point of view; all queries related to anthropological study should be made to corresponding author V.M.-T.

Ancient DNA wet laboratory work

The ancient DNA generation methods we used are referenced here in the main text and specified on a per-library basis in table S1. For the specialized extraction procedures we used for the soft tissue remains, we tried four different extraction buffers for each sample: buffer 1: 0.45 M EDTA (pH 8.0), 0.05% Tween 20, and proteinase K (0.25 mg/ml) (10); buffer 2: 4.2 M guanidine isothiocyanate, 0.053 M tris-HCl (pH 7.5), 0.0106 M EDTA, 2.12% sarkosyl, and proteinase K (0.2 mg/ml) (11); buffer 3: 0.01 M tris-HCl (pH 8.0), 0.01 M NaCl, 5 mM CaCl_2 , 2.5 mM EDTA (pH 8.0), 2% (w/v) SDS, 0.04 M dithiothreitol, and 10% proteinase K solution (>600 mAU/ml; QIAGEN)

(12); buffer 4: ATL buffer and 3% proteinase K solution, both from the DNeasy Blood and Tissue Kit (QIAGEN) (13).

Proteinase K was added just before adding buffer to samples. Approximately 37 mg of tissue was cut into small fragments (about 5 mm³) and homogenized in 750 µl of the selected extraction buffer using a pestle in a 2-ml Eppendorf low-bind DNA tube. The samples were incubated overnight at 56°C (rotated). DNA extraction followed previously published protocols for different lysis buffers (10–13), with one modification: Silica beads were used for purification instead of columns. Double-stranded libraries were prepared from the DNA extracts following an established protocol (14). To increase sequence coverage, single-stranded libraries were generated for most extracts, including from bone (15, 40). To reduce damage-induced errors, libraries were treated with uracil-DNA glycosylase to cleave ancient DNA molecules at uracils on the 5' end, a damage modality characteristic of ancient DNA (14, 45). Libraries were then enriched for sequences overlapping the mitochondrial genome and approximately 1.4 million genome-wide SNPs (16). Double-stranded libraries underwent one round of capture, and single-stranded libraries underwent two rounds, using the reagents and buffers produced by Twist Biosciences (16). Successful double-stranded libraries were sequenced on the Illumina NovaSeq S4 platform with paired-end reads, while single-stranded libraries were sequenced on the Illumina NovaSeq X platform, also with paired-end reads.

Radiocarbon dating

We obtained eight accelerator mass spectrometry radiocarbon dates for seven distinct individuals from the Pennsylvania State University Radiocarbon Laboratory. To generate these dates, we sonicated bone samples in successive washes of American Chemical Society–grade methanol, acetone, and dichloromethane for 30 min each at room temperature and followed this by three washes in Nanopure water to remove possible contaminants (including conservants and adhesives). We extracted bone collagen and purified it using a modified Longin method with ultrafiltration (>30-kDa gelatin) (46). We measured carbon and nitrogen concentrations and C/N ratios of the extracted and purified collagen samples using a Costech elemental analyzer (ECS 4010) and evaluated sample quality by percentage of crude gelatin yield, percentage of C, percentage of N, and C/N ratios before accelerator mass spectrometry ¹⁴C dating. C/N ratios for all samples fell between 3.1 and 3.3, indicating excellent preservation (47). We combusted collagen samples for 3 hours at 900°C in vacuum-sealed quartz tubes with CuO and Ag wires. We reduced sample CO₂ to graphite at 550°C using H₂ and a Fe catalyst, and we drew off reaction water with Mg(ClO₄) (48).

We pressed graphite samples into targets in aluminum boats, loaded them onto a target wheel, and performed all measurements using a modified National Electronics Corporation compact spectrometer with a 0.5 MV accelerator (NEC 1.5SDH-1). We corrected ¹⁴C ages for mass-dependent fractionation with measured $\delta^{13}\text{C}$ values (49) and compared these with samples of whale bone from the Pleistocene (>48,000 B.P.), bison bone from the late Holocene (around 1850 cal yr B.P.), cow bone from the late 1800s CE, and OX-2 oxalic acid standards. We calibrated radiocarbon ages using OxCal v.4.45 and the IntCal20 Northern Hemisphere curve (50, 51). For one individual (I36568), we generated two overlapping dates using different bone elements and used the RCombine function in OxCal to combine these into a single weighted average. We report the 95.4% Bayesian credible intervals for all dates (table S2).

Supplementary Materials

The PDF file includes:

Legends for tables S1 to S9

Other Supplementary Material for this manuscript includes the following:

Tables S1 to S9

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- Acknowledgments:** The research team in the field was composed of V.M.-T., Q.D., S.D., S. Sunil, and S. Singh. We are grateful for support from the beginning of the project from K. M. Khan, K. S. Khan, and K. S. Khan and also K. A. Khan, K. A. Khan, T. L. Tshangspa, S. Wazir, V. Mehta, and S. Choldan (Gasha-pa). We are grateful for the administrative support of K. M. Khan (Cultural Secretary, UT Ladakh), S. S. Jamwal (Additional Director General of Police, UT Jammu & Kashmir), K. I. Khan (Deputy Superintendent of Police, Leh), S. Sukhadeve (District Commissioner, Kargil), S. B. Suse (District Commissioner, Leh), and A. Lavasa (previous District Commissioner, Leh). We are thankful for the help in the field of I. Mohammad (Pata-pa), S. Ali (Pata-pa), A. Hussain (Shanak-pa), A. Sher (Khan-pa), J. Hussain (Pata-pa), M. Bano (Dengo-ma), M. Amin (Dengo-pa), S. Fatima (Dengo-ma), and M. Mussa (Ayid-pa). We appreciate discussions with S. B. de Moura, N. Tournadre, G. Jacques, A. Beylier, and P. Sebillaud. We are grateful to L. Kumar and N. Rai for sharing the Minero data. We thank D. Tabin for advice regarding Turkic and Mongolian history and suggestions for populations for coanalyzing from the Amur River region and from Tibet. We thank K. Sirak for help in interpreting radiocarbon dates. The accepted version of this article (before the editing, proofreading, and formatting changes following the paper being accepted) is subject to the Howard Hughes Medical Institute (HHMI) Open Access to Publications policy; HHMI laboratory heads have previously granted a nonexclusive CC BY 4.0 license to the public and a sublicensable license to HHMI in their research articles. Pursuant to those licenses, the accepted manuscript can be made freely available under a CC BY 4.0 license immediately on publication. **Funding:** We are grateful to the National Geographic society for support for the archaeological work. The ancient DNA data generation and analysis was supported by the National Institutes of Health (R01-HG012287); the John Templeton Foundation (grant 61220); by a private gift from J.-F. Clin; by the Allen Discovery Center program, a Paul G. Allen Frontiers Group advised program of the Allen Family Philanthropies; and by the Howard Hughes Medical Institute (to D.R.). **Author contributions:** Conceptualization: N.P., V.M.-T., Q.D., S.D., and D.R. Supervision: N.P. and D.R. Archaeological material: V.M.-T., Q.D., and S.D. Methodology: N.P., L.Q., and S.D. Data curation: N.P., S.D., G.S., S.M., and D.R. Validation: N.P., S.D., G.S., S.M., N.R., and D.R. Formal analysis and investigation: N.P., Q.D., S.D., L.Q., G.S., N.R., and S.M. Resources: V.M.-T., Q.D., S.M., N.R., and D.R. Software: N.P., S.M., and G.S. Funding acquisition: V.M.-T. and D.R. Project administration: N.R. and D.R. Contextualization: V.M.-T. and Q.D. Visualization: N.P. and D.R. Writing: N.P., V.M.-T., Q.D., S.D., L.Q., S.M., and D.R. **Competing interests:** The authors declare that they have no competing interests. **Data, code, and materials availability:** All data and code needed to evaluate and reproduce the results in the paper are present in the paper and/or the Supplementary Materials. Newly reported ancient sequencing data in this study are deposited at the European Nucleotide Archive (ENA) at the accession number PRJEB114260. Open science principles require making all data used to support the conclusions of a study maximally available, and we support these principles here by making fully publicly available not only the digital copies of molecules (the uploaded sequences) but also the molecular copies (the ancient DNA libraries themselves, which constitute molecular data storage). Those researchers who wish to carry out deeper sequencing of libraries published in this study should make a request to the corresponding author D.R. (reich@genetics.med.harvard.edu). We commit to granting reasonable requests as long as the libraries remain preserved in our laboratories, with no requirement that we be included as collaborators or coauthors on any resulting publications. The remaining skeletal samples are housed at Deccan College, and we commit to granting reasonable requests to study them from an anthropological point of view; all queries related to anthropological study should be made to corresponding author V.M.-T. (vmshrif@gmail.com).

Submitted 10 August 2025

Accepted 15 June 2026

Published 24 July 2026

10.1126/sciadv.aeb3636

Ancient genomes from Ladakh reveal 2800-year-old admixture between Tibetans and South Asians

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Sci. Adv. **12** (30), eaeb3636. DOI: 10.1126/sciadv.aeb3636

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<https://www.science.org/doi/10.1126/sciadv.aeb3636>

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