

# Mitochondrial genome evidence reveals successful Late Paleolithic settlement on the Tibetan Plateau

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Due to its numerous environmental extremes, the Tibetan Plateau—the world's highest plateau—is one of the most challenging areas of modern human settlement. Archaeological evidence dates the earliest settlement on the plateau to the Late Paleolithic, while previous genetic studies have traced the colonization event(s) to no earlier than the Neolithic. To explore whether the genetic continuity on the plateau has an exclusively Neolithic time depth, we studied mitochondrial DNA (mtDNA) genome variation within 6 regional Tibetan populations sampled from Tibet and neighboring areas. Our results confirm that the vast majority of Tibetan matrilineal components can trace their ancestry to Epipaleolithic and Neolithic immigrants from northern China during the mid-Holocene. Significantly, we also identified an infrequent novel haplogroup, M16, that branched off directly from the Eurasian M founder type. Its nearly exclusive distribution in Tibetan populations and ancient age (>21 kya) suggest that M16 may represent the genetic relics of the Late Paleolithic inhabitants on the plateau. This partial genetic continuity between the Paleolithic inhabitants and the contemporary Tibetan populations bridges the results and inferences from archaeology, history, and genetics.

mtDNA | origin

The Tibetan Plateau is characteristic of most extreme environmental conditions, with high absolute elevation, low temperature, extreme aridity, and hypoxia. Nonetheless, modern humans settled on this plateau by the Paleolithic Age. A number of Paleolithic sites excavated throughout the Tibetan Plateau have been dated to >20 thousand years ago (kya) [Fig. 1 and supporting information (SI) Table S1] (1–3), documenting the earliest human presence on the plateau well before the last glacial maximum (LGM, 22–18 kya). In contrast, evidence from classical genetic studies on the contemporary indigenous Tibetan population argues for a northern East Asian origin during the Neolithic (4), a scenario that seems compatible with the available historic records. According to the *Xin Tang Shu* (New Tang Annals; 11th century A.D.), proto-Tibetans (“Bo” people) can in fact trace their ancestry to the Di-Qiang, an ancient tribe that resided in northwest China about 3 kya (5). One possibility is that the Late Paleolithic settlers might have been eliminated due to exacerbated environmental conditions during the LGM or the Younger Dryas (12.8–11.6 kya), or were largely, if not completely, replaced by the Neolithic immigrants. This notion receives some support from archaeological observations; in particular, the main type of Neolithic tools excavated on the plateau, microliths, show typical features of the northern Chinese tool culture (6). However, these microliths also display some characteristics of the Tibetan paleoliths (7, 8). This mosaic

feature raises another possibility that the Neolithic immigrants had received some contribution from the Paleolithic settlers through either cultural or demic contact.

Based on the genetic evidence obtained so far from Y chromosome (9, 10) and mitochondrial DNA (mtDNA) (11–13) data, the majority of Tibetan genetic components can trace their origins to the Neolithic immigrants from northern East Asia. No solid genetic evidence indicates the existence of any ancient genetic relics from Paleolithic settlers. Nearly all of the Y chromosome markers in Tibetans analyzed recently (14) are indeed suggestive of more recent genetic inflow, except for the paralog O3a5\*-M134 (comprising the O3a5-M134 Y chromosomes not belonging to O3a5a-M117) which has a more ancient age of 22 kya. The high frequency of haplogroup D-M174 (the Eurasian YAP+ founder haplogroup) in Tibetans had previously led some researchers to propose an additional genetic contribution from Central Asians (9) or to infer an ancient relationship between Tibetans and Japanese (15).

One must concede that most of those genetic studies were hampered by either limited resolution of the classification tree (9, 11, 13), relatively small sample sizes (9–12), or, most importantly, potentially biased sampling coverage, in that most of the Tibetan samples came from the peripheral regions of Tibet, including Yunnan and Qinghai Provinces (9, 10, 12, 13) or from an undifferentiated “general population” (14). Consequently, phylogeographic analyses performed on Tibetans were only rudimentary and proved largely inconclusive, as fine-scale founder types could not be identified.

## Results and Discussion

To investigate at a finer scale whether any genetic relics from the Paleolithic inhabitants have survived in the modern Tibetan population, we analyzed 680 individuals, representing 6 popu-

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The authors declare no conflicts of interest.

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Data deposition: All of the sequences obtained in the present study have been deposited into GenBank, with accession numbers FJ544230-FJ544243, FJ968772-FJ968775, and GU014563-GU014569 (for whole mtDNA genomes) and FJ543469-FJ544148 (for control region sequences).

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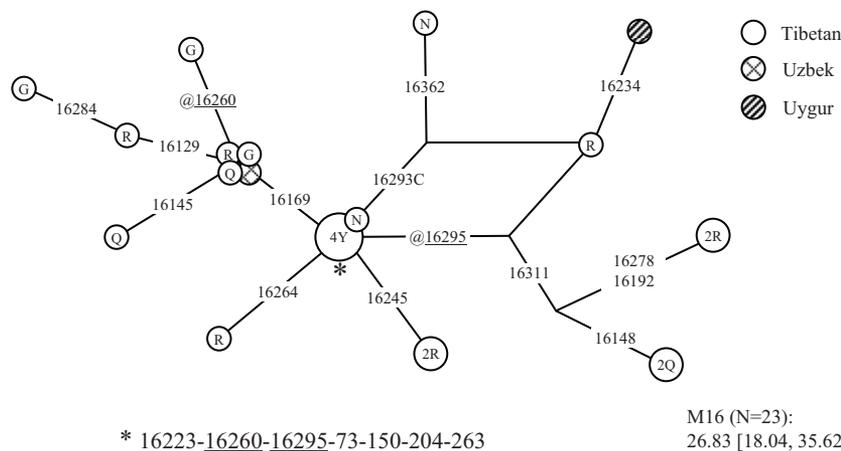
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**Fig. 5.** Constructed median network displaying the control region information of M16 lineages. This network was constructed manually according to Bandelt et al. (31). The data used here were collected from the literature (Table S2) and the present study (Table S3). The sequence information used for network construction was confined to segment 16047-16497. Time estimation was carried out based on segment 16051-16400 as described previously (35). The asterisk denotes the ancestral node of the haplogroup defined by motif 16223-16260-16295-73-150-204-263. See the legend of Fig. 3 for more information.

Our findings have significant implications for the seemingly conflicting inferences drawn from archaeology, genetics, and historic records. Essentially, the previous debate on the peopling of the Tibetan Plateau concerned the issue of whether or not the initial Late Paleolithic inhabitants on the plateau were *completely* replaced by the later Neolithic immigrants. In this study, the observed genetic continuity between the initial Paleolithic inhabitants and the modern populations on the Tibetan Plateau strongly suggests that modern humans did exist on the plateau before the LGM, and it is these Paleolithic people who have successfully overcome the extremely harsh climate and environments and made some genetic contribution (albeit limited) to the contemporary inhabitants. This also helps to explain why the excavated microliths on the Tibetan Plateau display mosaic features of both northern Chinese tool culture (6) and the Tibetan Paleoliths (7, 8).

In summary, although the vast majority of identified mtDNA lineages found in Tibetans can trace their origins to northern East Asia and may have entered the Tibetan Plateau in the

Holocene, our study provides support for the existence of genetic relics of the Late Paleolithic settlers in Tibetans, indicating some genetic continuity between the initial Paleolithic inhabitants and the modern populations on the Tibetan Plateau. Our findings may contribute to resolving the long-standing debates among the fields of archaeology, history, and genetics.

### Subjects and Methods

**Sampling.** Blood samples from 680 unrelated individuals of 6 Tibetan populations were collected with informed consent. Total DNA was extracted by the standard phenol/chloroform method. The populations were labeled as follows: Nakchu-Tibetans, 168 Tibetans from Nakchu Prefecture of Tibet; Shigatse-Tibetans, 220 Tibetans from Shigatse Prefecture of Tibet; Yunnan-Tibetans, 71 Tibetans from Diqing Tibetan Autonomous Prefecture of Yunnan Province; Qinghai-Tibetans, 76 Tibetans from Qinghai Province; Sichuan-Tibetans, 62 Tibetans from Liangshan Yi Autonomous Prefecture of Sichuan Province; and Gansu-Tibetans, 83 Tibetans from Gannan Tibetan Autonomous Prefecture of Gansu Province.

**Sequencing and RFLP Typing.** With the exception of 37 Qinghai-Tibetans, for which only the segment spanning from position 16001 to position 16497 (relative to the revised Cambridge reference sequence, rCRS) (20, 21) was amplified and sequenced as described elsewhere (22), the entire mtDNA control region for the other 643 samples was amplified, sequenced, and dealt with as described previously (23), with minor modifications in the reverse primers [i.e., replacement of the previous reverse primer H408 by H902 (5'-GACTTGGGTTAATCGTGTGAC-3') or H575 (5'-TGAGGAGGTAAGCTACATA-ACTG-3'), to cover more informative sites, such as position 489]. To confirm the haplogroup status inferred from the control region motifs, the following coding region sites were selected for typing by either RFLP or DNA sequencing [according to the reconstructed East Asian mtDNA tree (19, 24)]: 10397*AluI* (for macrohaplogroup M), 5176*AluI*/4883 (D), 3008*TaqI*/3010 (D4), 4831*HhaI*/

**Table 2. Comparisons of nonsynonymous and synonymous substitutions between M16 and the other East Asian M lineages**

Gene	M16		East Asian M lineages*		<i>P</i> <sup>S</sup>
	NS <sup>†</sup>	S <sup>‡</sup>	NS <sup>†</sup>	S <sup>‡</sup>	
ND1	1	3	9	15	1.000
ND2	2	1	8	15	0.538
COX1	0	4	3	21	1.000
COX2	2	1	5	17	0.180
ATP8	0	1	2	5	1.000
ATP6	2	0	6	6	0.473
COX3	0	0	6	8	1.000
ND3	0	2	1	3	1.000
ND4L	0	0	0	8	1.000
ND4	0	4	7	15	0.546
ND5	1	3	9	24	1.000
ND6	0	1	4	11	1.000
CytB	2	4	14	13	0.656
Totally	10	24	74	161	1.000

\*Data from refs. 19 and 24. Haplogroups M9a, M13, and G3a1, which are prevalent in Tibetans, were not considered.

<sup>†</sup>NS refers to the number of nonsynonymous substitutions.

<sup>‡</sup>S refers to the number of synonymous substitutions.

<sup>S</sup>*P* values determined by the 2-tailed Fisher's exact test.

**Table 3. Comparison of internal and terminal NS/S on the East Asian mtDNA tree**

	<i>n</i> <sup>*</sup>	NS <sub>i</sub> /S <sub>i</sub> <sup>†</sup>	NS <sub>t</sub> /S <sub>t</sub> <sup>†</sup>	<i>P</i>
M16	13	0.78 (7/9)	0.20 (3/15)	0.13
East Asian M lineages <sup>‡</sup>	38	0.56 (24/43)	0.42 (50/118)	0.44
<i>P</i>		0.58	0.29	

\*Sample size.

<sup>†</sup>Indices "i" and "t" refer to the corresponding fractions of NS/S (see Table 2) for the internal branches and terminal branches, respectively, of the East Asian mtDNA tree. *P* values were obtained by the 2-tailed Fisher's exact test.

<sup>‡</sup>Data from refs. 19 and 24. Haplogroups M9a, M13, and G3a1, which are prevalent in Tibetans, were not considered.

4833 (G), 9820HinfI/6455 (M7), 6680 (M7b), 4715 (M8), 14465AclI (M8a), 13262AluI (C), 3391HaeIII/3394 (M9a), 12549 (M10), 10644RsaI (M10a), 7641AluI (M11), 12030 (M12), 6023/6253 (M13), 663HaeIII/663 (A), 5417 (N9), 10310 (F), 12406HincII (F1), 14766 (HV), 7025AluI (H), and 8281–8289del (B). For some Tibetan samples [i.e., 32 Yunnan-Tibetans and 8 Qinghai-Tibetans, for which segments from 16001 to 16497 have been reported by Yao et al. (12)], the corresponding HVS-II segments and some further coding region sites were sequenced and/or screened as well.

**Data Analyses.** Based on the combined control region and coding region information, the majority of the samples were unambiguously assigned to haplogroups under the guidance of the reconstructed mtDNA trees of East Asian (19, 24) and South Asian (25, 26) mtDNA lineages. For those Tibetan mtDNAs that remained unassignable, complete mtDNA genome sequencing was performed, as described previously (27), to fully determine their exact phylogenetic status. Specifically, the whole mtDNA genome was amplified in 4 overlapping fragments by using 4 pairs of primers (L13894/H2187, L16777/H6505, L5868/H10718, and L9877/H14676), then each fragment was sequenced using a set of inner primers. (See table 1 in ref. 27 for detailed information on the primers.) With this approach, the previously reported problems in mtDNA genome datasets, such as artificial recombination (28) and amplification of pseudomitochondrial gene (29), could be minimized. Two samples (Uzb57 and Uyg5) reported with M16 control region variation motif (16) (GenBank accession numbers AY678062 and AY678009) were also selected for complete sequencing.

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The haplogroup allocations of the reported mtDNA data from the literature (Table S2) were reevaluated by the near-matching strategy (30). The reduced median network for each haplogroup was constructed manually [as described by Bandelt et al. (31)] and then confirmed using Network 4.510 (<http://www.fluxus-engineering.com/sharenet.htm>). The time to the most recent common ancestor of a haplogroup was estimated as described previously (32–35). PCA was conducted as described previously (30).

The Chinese M sequences used in our comparative analysis of nonsynonymous and synonymous substitution were obtained from the literature (19, 24). Three sequences (GenBank accession numbers AY255153, DQ272115, and DQ272108) belonging to M9a, M13, and G3a1, respectively, were disregarded, because these haplogroups are very frequent in Tibetans and thus might have suffered similar high-altitude selection pressure as M16 did. Mutations were classified into nonsynonymous and synonymous substitutions for each gene using mtDNA-GeneSyn software (36). Each mutation was classified as internal or terminal on the mtDNA tree, as described previously (37). Fisher's exact test was used to examine the difference in each gene between M16 and the other East Asian M lineages.

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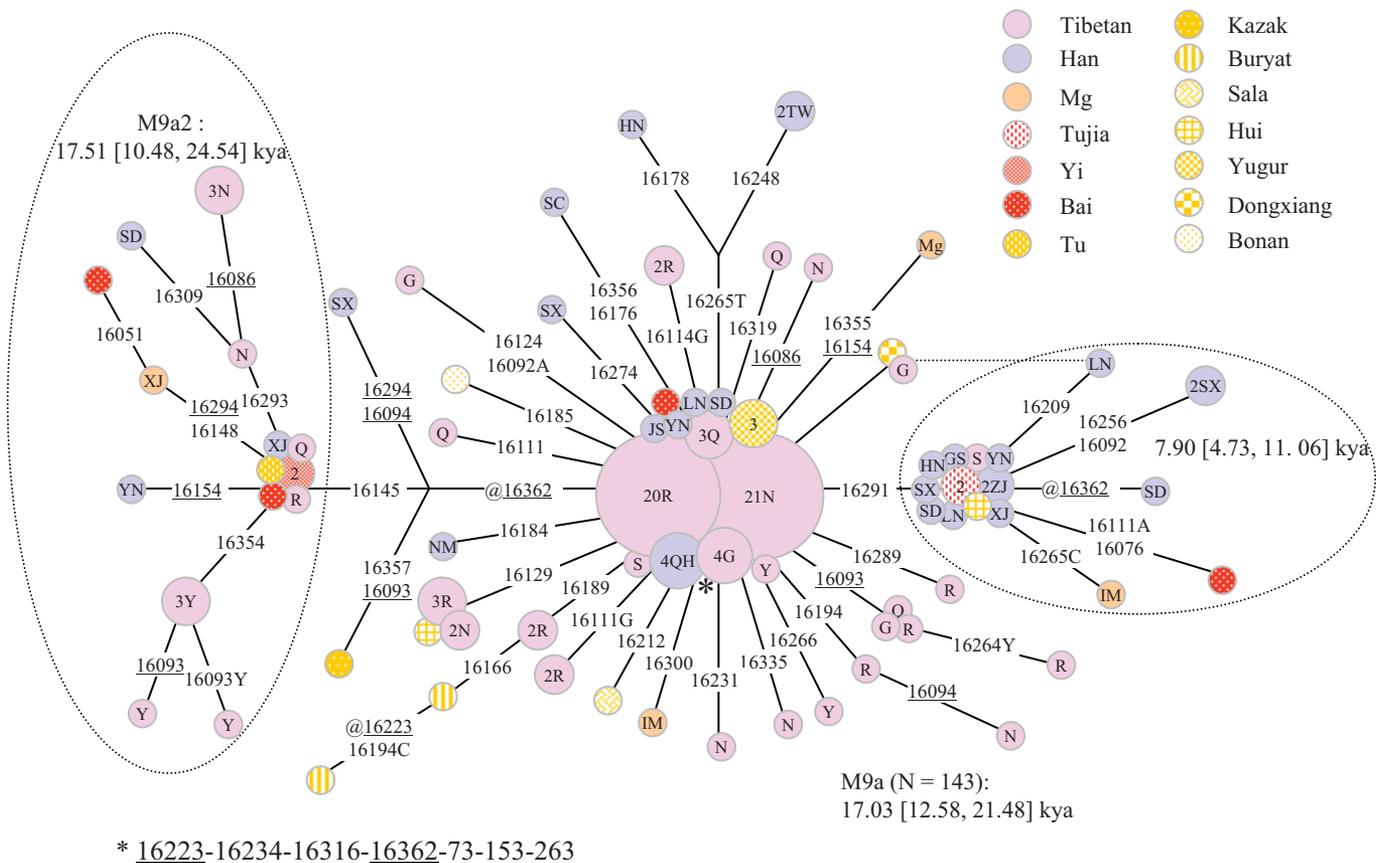
# Supporting Information

Zhao et al. 10.1073/pnas.0907844106

## SI Text

1. Andrews RM, et al. (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat Genet* 23:147.
2. Kong Q-P, et al. (2003) Phylogeny of East Asian mitochondrial DNA lineages inferred from complete sequences. *Am J Hum Genet* 73:671–676.
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**Fig. S2.** Median network of haplogroup M9a. This network was constructed manually according to Bandelt et al. (7). The data used here were collected from the literature (Table S2) and the present study (Tables S3 and S5). The sequence information used for network construction was confined to segment 16047–16497. Suffixes “A,” “C,” “G,” and “T” refer to transversions; “Y” specifies heteroplasmic status *CT* at the site; recurrent mutations are underlined; and “@” denotes a reverse mutation. Time estimation was carried out based on segment 16051–16400 as described previously (8). Codes “N,” “R,” “Q,” “Y,” “S,” and “G” refer to sampling locations (Nakchu, Shigatse, Qinghai, Yunnan, Sichuan, and Gansu, respectively) of different regional Tibetan populations. The asterisk denotes the ancestral node of the haplogroup defined by motif 16223-16234-16316-16362-73-153-263.









**Table S1. Relevant information of the excavation sites on the Tibetan Plateau.**

Label on Figure 1	Excavation Site	Estimated Date (kya)	Method for Date Estimation	Location	References
a	Pulan	10-50	Comparison of stoneware	Ali, Tibet (3700 m. a. s. l.)	(1)
b	Quesang	18-24	Optically stimulated luminescence	Lhasa, Tibet (4200 m. a. s. l.)	(2)
c	Selincuo	30-40	Radiocarbon dating	Bange, Nakchu, Tibet (4600 m. a. s. l.)	(3)
d	Duogeze	10-50	Comparison of stoneware	Shenza, Nakchu, Tibet (4830 m. a. s. l.)	(4)
e	Zhuluole	10-50	Comparison of stoneware	Shenza, Nakchu, Tibet (4800 m. a. s. l.)	(4)
f	Sure	10-50	Comparison of stoneware	Dingri, Shigatse, Tibet (4500 m. a. s. l.)	(4)
g	Luling	10-50	Comparison of stoneware	Shenza, Nakchu, Tibet (4700 m. a. s. l.)	(4)
h	Hadongtang & Quedetang	10-50	Comparison of stoneware	Jilong, Shigatse, Tibet (4100 m. a. s. l.)	(4)
i	Zhabu	10-50	Comparison of stoneware	Ritu, Ali, Tibet (4500 m. a. s. l.)	(4)
j	Xiadacuo	10-50	Comparison of stoneware	Ritu, Ali, Tibet (4500 m. a. s. l.)	(5)
k	Heimahe & Jiangxigou	11-15	Radiocarbon dating	Gonghe, Hainan Tibetan Autonomous Prefecture, Qinghai (3210 m. a. s. l.; 3330 m. a. s. l.)	(6)
l	XiaoQaidam	30-36	Radiocarbon dating of ostracods	Haixi Mongolian and Tibetan Autonomous Prefecture, Qinghai (3100 m. a. s. l.)	(4)

Notes: m. a. s. l. = meters above sea level.

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**Table S2. Populations under study**

Code	G*	Population	Language Affiliation <sup>†</sup>	N <sup>‡</sup>	Sample Location	References
1	NE	Daur	Mongolic	45	Inner Mongolia	(1)
2	NE	Ewenki	Tungusic	47	Inner Mongolia	(1)
3	NE	Oroqen	Tungusic	44	Inner Mongolia	(1)
4	NE	Kazak	Turkic	53	Kashen, Xinjiang	(2, 3)
5	NE	Uzbek	Turkic	58	Yili, Xinjiang	(2)
6	NE	Uygur	Turkic	47	Yili/Kashen, Xinjiang	(2, 3)
7	NE	Mongolian_1	Mongolic	48	Inner Mongolia	(1)
8	NE	Mongolian_2	Mongolic	49	Yili, Xinjiang	(2)
9	NE	Korean_1	Koreanic	48	Inner Mongolia	(1)
10	NE	Korean_2	Koreanic	55	Yan Bian, Jilin	(4)
11	NE	Hui	Sinitic	45	Yili, Xinjiang	(2)
12	NE	Dongxiang	Mongolic	96	Gansu	(5)
13	NE	Bonan	Mongolic	95	Gansu	(6)
14	NE	Yugur	Turkic / Mongolic	100	Gansu	(7)
15	NE	Sala	Turkic	99	Qinghai	(8)
16	TB	Pumi	Tibeto-Burman	35	Ninglang, Lijiang, Yunnan	(9)
17	TB	Naxi	Tibeto-Burman	45	Lijiang, Yunnan	(9)
18	SE	Va_1	Tai-Kadai	22	Simao, Yunnan	(10)
19	SE	Va_2	Tai-Kadai	36	Ximeng/Gengma, Simao, Yunnan	(11)
20	TB	Tujia_1	Tibeto-Burman	66	Western Hunan	(9)
21	TB	Tujia_2	Tibeto-Burman	31	Yongshun, Hunan	(9)
22	TB	Yi_1	Tibeto-Burman	40	Shuangbai, Yunnan	(9)
23	TB	Yi_2	Tibeto-Burman	16	Xishuangbanna, Yunnan	(9)
24	SE	Dai_1	Tai-Kadai	21	Xishuangbanna, Yunnan	(10)
25	SE	Dai_2	Tai-Kadai	38	Xishuangbanna, Yunnan	(12)
26	TB	Aini	Tibeto-Burman	47	Xishuangbanna, Yunnan	(9)
27	TB	Bai_1	Tibeto-Burman	40	Dali, Yunnan	(9)
28	TB	Bai_2	Tibeto-Burman	19	Xishuangbanna, Yunnan	(9)
29	TB	Hani	Tibeto-Burman	33	Xishuangbanna, Yunnan	(9)
30	TB	Jino	Tibeto-Burman	18	Xishuangbanna, Yunnan	(9)
31	TB	Lahu_1	Tibeto-Burman	15	Xishuangbanna, Yunnan	(9)
32	TB	Lahu_2	Tibeto-Burman	32	Simao, Yunnan	(10)
33	TB	Lahu_3	Tibeto-Burman	35	Lancang, Simao, Yunnan	(11)
34	SE	Taiwan Aborigines	Formosan, Austronesian	180	Taiwan	(13)
35	SH	Han_Taiwan	Sinitic	155	Taiwan	(14)
36	SH	Han_Anhui	Sinitic	42	Hefei, Anhui	(15)
37	SH	Han_Fujian	Sinitic	54	Changtin, Fujian	(15)
38	SH	Han_Guangdong_1	Sinitic	30	Zhanjiang, Ganddong	(16)
39	SH	Han_Guangdong_2	Sinitic	69	Guangdong	(17)
40	NH	Han_Gansu	Sinitic	45	Gansu	(15)
41	SH	Han_Guangxi	Sinitic	26	Tianlin, Guangxi	(15)
42	SH	Han_Hubei	Sinitic	42	Wuhan, Hubei	(16)
43	SH	Han_Hunan_1	Sinitic	82	Changsha, Hunan	(18)
44	SH	Han_Hunan_2	Sinitic	16	Changsha, Hunan	(15)
45	NH	Han_Jilin	Sinitic	51	Yan Bian, Jilin	(19)
46	SH	Han_Jiangsu	Sinitic	67	Nanjing, Jiangsu	(15)

Code	G*	Population	Language Affiliation <sup>†</sup>	N <sup>‡</sup>	Sample Location	References
47	SH	Han_Jiangxi	Sinitic	23	Nanchang, Jiangxi	(15)
48	NH	Han_Liaoning_1	Sinitic	51	Fengcheng, Liaoning	(16)
49	NH	Han_Liaoning_2	Sinitic	51	Dalian, Liaoning	(15)
50	NH	Han_Neimeng	Sinitic	45	Chifeng, Neimeng	(15)
51	NH	Han_Qinghai	Sinitic	44	Xining, Qinghai	(15)
52	SH	Han_Sichuan	Sinitic	70	Weicheng, Sichuan	(15)
53	NH	Han_Shandong_1	Sinitic	50	Qingdao, Shandong	(16)
54	NH	Han_Shandong_2	Sinitic	76	Tai'an, Shandong	(20)
55	SH	Han_Shanghai	Sinitic	56	Shanghai	(15)
56	SH	Han_Shanghai	Sinitic	120	Shanghai	(21)
57	NH	Han_Shaanxi_1	Sinitic	85	Xi'an, Shaanxi	(18)
58	NH	Han_Shaanxi_2	Sinitic	53	Xi'an, Shaanxi	(15)
59	NH	Han_Xinjiang	Sinitic	47	Yili, Xinjiang	(16)
60	SH	Han_Yunnan_1	Sinitic	43	Kunming, Yunnan	(16)
61	SH	Han_Yunnan_2	Sinitic	58	Huize, Yunnan	(15)
62	SH	Han_Zhejiang	Sinitic	61	Hangzhou, Zhejiang	(15)
63	TB	Nakchu_Tibetan	Tibetic	168	Nakchu, Tibet	Present study
64	TB	Shigatse_Tibetan	Tibetic	220	Shigatse, Tibet	Present study
65	TB	Tibet_Tibetan	Tibetic	54	Tibet	(22)
66	TB	Qinghai_Tibetan_1	Tibetic	56	Qinghai	(9)
67	TB	Qinghai_Tibetan_2	Tibetic	76	Qinghai	Present study
68	TB	Yunnan_Tibetan_1	Tibetic	35	Zhongdian, Diqing, Yunnan	(9)
69	TB	Yunnan_Tibetan_2	Tibetic	24	Diqing, Yunnan	(10)
70	TB	Yunnan_Tibetan_3	Tibetic	71	Diqing, Yunnan	Present study
71	TB	Sichuan_Tibetan	Tibetic	62	Liangshan, Sichuan	Present study
72	TB	Gansu_Tibetan	Tibetic	83	Gannan, Gansu	Present study

Notes: \* Abbreviations “NE”, “TB”, “SE”, “NH” and “SH” refers to the northern ethnic groups,

Tibeto-Burman speaking populations, southern (autochthonous) ethnic groups, Northern Han

Chinese and Southern Han Chinese, respectively; <sup>†</sup> Classified according to the Ethnologue:

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