

# A Mitochondrial Revelation of Early Human Migrations to the Tibetan Plateau Before and After the Last Glacial Maximum

Zhendong Qin,<sup>1</sup> Yajun Yang,<sup>1,2</sup> Longli Kang,<sup>1,3</sup> Shi Yan,<sup>4</sup> Kelly Cho,<sup>5</sup> Xiaoyun Cai,<sup>1</sup> Yan Lu,<sup>1</sup> Hongxiang Zheng,<sup>1</sup> Dongchen Zhu,<sup>1</sup> Dongmei Fei,<sup>1</sup> Shilin Li,<sup>1</sup> Li Jin,<sup>1,2,4\*</sup> Hui Li<sup>1\*</sup>, and The Genographic Consortium

<sup>1</sup>State Key Laboratory of Genetic Engineering and Ministry of Education Key Laboratory of Contemporary Anthropology, School of Life Sciences and Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, China

<sup>2</sup>China Medical City Institute of Health Sciences, Taizhou, Jiangsu 225300, China

<sup>3</sup>Department of Medicine, Tibet Nationality College, Xianyang, Shaanxi 712082, China

<sup>4</sup>Chinese Academy of Sciences and Max Planck Society Partner Institute for Computational Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

<sup>5</sup>Department of Genetics, Yale School of Medicine, New Haven, CT 06520

**KEY WORDS** mitochondrial DNA; complete sequence; the Tibetan Plateau; last glacial maximum

**ABSTRACT** As the highest plateau surrounded by towering mountain ranges, the Tibetan Plateau was once considered to be one of the last populated areas of modern humans. However, this view has been tremendously changed by archeological, linguistic, and genetic findings in the past 60 years. Nevertheless, the timing and routes of entry of modern humans into the Tibetan Plateau is still unclear. To make these problems clear, we carried out high-resolution mitochondrial-DNA (mtDNA) analyses on 562 Tibeto-Burman inhabitants from nine different regions across the plateau. By examining the mtDNA haplogroup distributions and their principal components, we demon-

strated that maternal diversity on the plateau reflects mostly a northern East Asian ancestry. Furthermore, phylogeographic analysis of plateau-specific sublineages based on 31 complete mtDNA sequences revealed two primary components: pre-last glacial maximum (LGM) inhabitants and post-LGM immigrants. Also, the analysis of one major pre-LGM sublineage A10 showed a strong signal of post-LGM population expansion (about 15,000 years ago) and greater diversity in the southern part of the Tibetan Plateau, indicating the southern plateau as a refuge place when climate dramatically changed during LGM. *Am J Phys Anthropol* 000:000–000, 2010. ©2010 Wiley-Liss, Inc.

The Tibetan Plateau, the “Roof of the World,” is the highest plateau on the earth with an average elevation of more than 4,000 m, covers more than 2,500,000 km<sup>2</sup>

of plateaus and mountains in central Asia and is surrounded by towering mountain ranges (Himalayas on the south, Karakoram on the west, Kunlun on the north-

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The Genographic Consortium includes: Janet S. Ziegler (Applied Biosystems, Foster City, California, United States); Pandikumar Swamikrishnan (IBM, Somers, New York, United States); Asif Javed, Laxmi Parida, Daniel E. Platt, and Ajay K. Royyuru (IBM, Yorktown Heights, New York, United States); Lluís Quintana-Murci (Institut Pasteur, Paris, France); R. John Mitchell (La Trobe University, Melbourne, Victoria, Australia); Danielle A. Badro, Marc Haber, and Pierre A. Zalloua (Lebanese American University, Chouran, Beirut, Lebanon); Syama Adhikarla, Arun Kumar Ganesh Prasad, Ramasamy Pitchappan, Kavitha Valampuri John, and Arun Varatharajan Santhakumari (Madurai Kamaraj University, Madurai, Tamil Nadu, India); Christoff J. Erasmus, Angela Hobbs, and Himla Soodyall (National Health Laboratory Service, Johannesburg, South Africa); Elena Balanovska and Oleg Balanovsky (Research Centre for Medical Genetics, Russian Academy of Medical Sciences, Moscow, Russia); Chris Tyler-Smith (The Wellcome Trust Sanger Institute, Hinxton, United Kingdom); Daniela R. Lacerda and Fabrício R. Santos (Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil); Pedro Paulo Vieira (Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil); Jaume Bertranpetit, David Comas, Begoña Martínez-Cruz, and Marta Melé (Universitat Pompeu Fabra, Barcelona, Spain); Christina J. Adler, Alan Cooper, Clio S. I. Der Sarkissian, and Wolfgang Haak (University of Adelaide, South Australia, Australia); Matthew E. Kaplan and Nirav C. Merchant (University of Arizona, Tucson, Arizona, United States); Colin

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\*Correspondence to: Li Jin or Hui Li, School of Life Sciences, Fudan University, Shanghai 200433, China.

E-mail: JinLi.Fudan@gmail.com or LiHui.Fudan@gmail.com

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west, and Qilian on the northeast). Despite its inhospitable environment, the Tibetan Plateau is now occupied by over seven million people (2000 census), mostly indigenous Tibetans. When and how modern human conquered the Tibetan Plateau is one of the most interesting questions of human evolution, however, little was known about the peopling of Tibet.

Some efforts have been made to estimate the age of the peopling of Tibet in the fields of archeology and linguistics. Archeological records of Late Paleolithic age dated the human presence on the plateau back to about 20–30 thousand years ago (KYA) (Aldenderfer and Yinong, 2004). However, the sparseness and discontinuity of archeological findings on this plateau have led to ambiguous explanations of the demographic history of indigenous Tibetans. In contrast to the old history of the Tibetans suggested by archeology, linguistic studies (Van Driem, 1998, 2001, 2002, 2005) postulated complex models and favored a much more recent Neolithic peopling of the plateau. To clarify the inconsistency of the timing by different disciplines, more solid evidences are required, such as genetic diversity in Tibet.

During the past two decades, genetic markers (especially mitochondrial and Y chromosomal markers) were proved extremely useful in tracing population history in East Asia (Ding et al., 2000; Jin and Su, 2000; Zhang et al., 2007). However, previous genetic studies on the Tibetans also issued inconsistent results. Some studies using classical genetic traits (Du et al., 1997), autosomal microsatellite markers (Gayden et al., 2009; Kang et al., 2010) and mitochondrial DNA (mtDNA) (Torroni et al., 1994) suggested a north Asian origin of Tibetans. While evidences from the Y chromosomal *Alu* insertion (YAP) marker revealed much more intricate stories for the origin of Tibetan peoples (Hammer et al., 1997; Qian et al., 2000; Su et al., 2000; Shi et al., 2008). The YAP polymorphism was enriched on the Tibetan Plateau, Japan, and Andaman islands, but almost absent in other regions. This contradiction has been attributed to different demographic histories between patrilineal and matrilineal genetic pools, but it is still highly debated concerning the resolution of the limited markers used and the incomplete sampling in each study. More detailed works about Tibetan populations both on mitochondrial DNA and Y chromosome DNA are definitely required.

Maternally inherited mtDNA plays an important role in studying modern human migrations (Wallace et al., 1999; Pakendorf and Stoneking, 2005) and probable selection effects (Ruiz-Pesini et al., 2004; Ingman and Gyllensten, 2007). Over the past few years, genetic studies about mtDNA of populations residing on and around the Tibetan Plateau have been conducted (Qian et al., 2001; Torroni et al., 1994; Wen et al., 2004b; Yao and Zhang, 2002), which led to a number of important insights into the genetic history of Tibetans and their adaptive process. However, most of these studies focused on the short sequence of mitochondrial control region, lacking power to distinguish detailed population histories. The recent development of mtDNA analysis based on complete sequencing made it possible to reconstruct maternal phylogeny of Tibetan populations thus may shed light on their genetic structure and population history. Gu et al. (2008, 2009) first used this analysis in comparing mtDNA genomes of Tibetans and Han Chinese and proposed possible selective effects in Tibetan populations. However, they did not have enough Tibetan population samples and therefore did not draw any con-

clusions on the origin of the Tibetans. Actually, those previous studies all suffered from insufficient population sample coverage of the plateau (a maximum of three population samples), lacking power of detecting substructure among Tibetan populations.

A recent article about mtDNA genome variation within six regional Tibetan populations has revealed successful Late Paleolithic settlement on the Tibetan Plateau (Zhao et al., 2009). In this study, we further sampled eleven Tibeto-Burman populations from nine different geographic regions of the Tibetan Plateau. On the basis of our increased population coverage and comprehensive mtDNA information with complete mtDNA sequences, we traced the origin and the expansion of Tibetan populations on the plateau. Our findings indicate that Tibetan maternal gene pool consists of both pre- and post-LGM components. In addition, we found a signal of post-LGM population expansion on the plateau.

## MATERIALS AND METHODS

### Population samples and DNA extraction

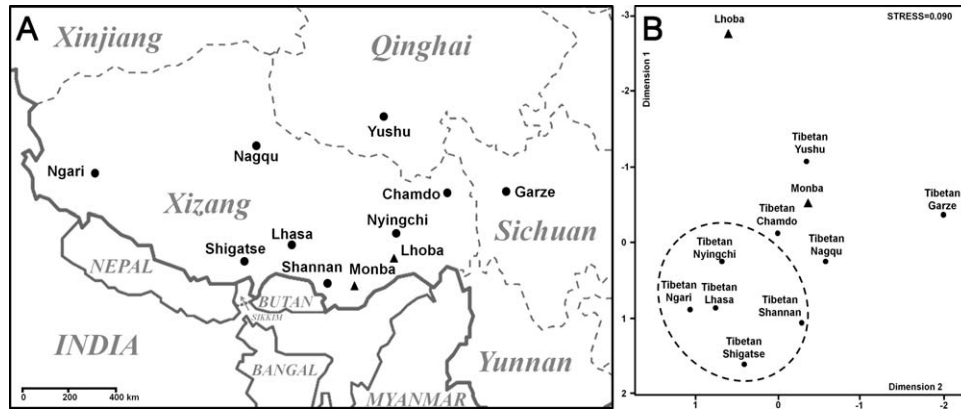
Samples were collected by buccal swab from 562 anonymous and unrelated volunteers living on in the Tibetan Plateau with appropriate informed consent under protocols approved by the relevant institutional review boards. These population samples included nine Tibetan populations (46 individuals from Ngari, 58 from Nagqu, 59 from Shigatse, 59 from Lhasa, 61 from Chamdo, 56 from Shannan, 53 from Nyingchi, 44 from Yushu of Qinghai, 55 from Garze of western Sichuan), one Monba population (51 from Nyingchi), and one Lhoba population (20 from Shannan). The sampling locations were illustrated in Figure 1A. Genomic DNA was extracted using Silica/GuSCN method (Supporting Information S1).

### Sequencing the control region and typing SNPs in the coding regions

The hypervariable segment I (HVS-I) of the control region was amplified by primers L15974 and H16488 (Yao et al., 2002). Purified PCR products were sequenced using the BigDye terminator cycle sequencing kit and an ABI 3130XL genetic analyzer (Applied Biosystems). Samples with a poly-cytosine (poly-C) tract caused by the transition at nucleic position (np) 16189 were sequenced in both directions. Overall, 562 HVS-I sequences have been submitted to GenBank. A SNaPshot assay was used for typing SNPs in the coding regions to resolve haplogroup determination. This assay was designed as a multiplex panel including 21 coding region SNPs and one length variation marker (for information of markers and primers see Supporting Information Table S2). All haplogroup F (10310G) samples were screened at np 12406 using enzyme *HpaI* digesting. Some newly defined haplogroup diagnostic SNPs were checked by directly sequencing of appropriate fragments. The mtDNA haplogroup nomenclature used here was following with PhyloTree.org mtDNA tree Build 6 (28 September, 2009) (van Oven and Kayser, 2009) and previous studies (Tanaka et al., 2004; Kong et al., 2003, 2006; Derenko et al., 2007; Soares et al., 2008).

### Sequencing whole mtDNA genome

Whole mtDNA genome sequencing was performed for a selection of 31 samples following methods described by



**Fig. 1.** A good correlation between the geographic locations and mtDNA HVS-I region diversities of the Tibetan samples. (A) Geographic locations of the 11 indigenous populations in the Tibetan plateau. (B) Two-dimensional MDS plot based on an  $F_{ST}$  distance matrix calculated from 440-bp-length sequences of mtDNA HVS-I region.

Torrioni et al. (2001). Sequencing trace files were edited and aligned with the DNASTAR software (DNASTAR, Inc.). Mutations were scored relative to the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999).

### Data analysis

For HVS-I sequence data; median joining networks (Bandelt et al., 1995) were constructed using the free software package Network 4.1 (Fluxus-engineering.com). Descriptive statistical indexes, the Tajima's  $D$  (Tajima, 1989) and Fu's  $F_s$  (Fu, 1997) neutrality tests, pairwise distances between populations ( $F_{ST}$ ), mismatch distribution, and analysis of molecular variance (AMOVA) (Excoffier et al., 1992) were calculated using Arlequin software (version 3.11) (Excoffier et al., 2005). The mutation model used here was Kimura two-parameter model with a Gamma shape parameter ( $\alpha$ ) of 0.26.

For complete sequences, we first constructed ML phylogenetic trees with PhyML3.0 (Ronquist and Huelsenbeck, 2003) under HKY+G mutation model with an alpha parameter of 0.12 (Macaulay et al., 2005) for 100 bootstrap runs. A consensus tree was obtained through these 100 bootstrap trees by CONSENSUS implemented in PHYLIP package (version 3.69) (Felsenstein, 1989). Then the assumption of a molecular clock was tested with the PAML package (Yang, 1997) under the same evolutionary model. The null hypothesis of a molecular clock cannot be rejected ( $P = 0.797$ ). The complete mtDNA phylogeny was constructed and verified with several runs using Network (version 4.5) (Fluxus-engineering.com). The highly variable site 16519 and the length variation in the poly-C stretches between np 16180–16193 and 309–315 were discarded for phylogeny construction. The coalescence times were estimated with the  $\rho$  statistics (Forster et al., 1996), and standard errors were calculated following Saillard et al. (2000). For estimating the time to the most recent common ancestor (TMRCA) of each cluster, we used Mishmar rate (Saillard et al., 2000) and modified Mishmar rate (Perego et al., 2009) for coding region sequences (from np 577 to 16023); modified Kivisild rate (Perego et al., 2009) and Soares synonymous rate for synonymous mutations; and Soares rate (Soares et al., 2009) for complete mitochondrial genomes (all the substitutions excluding the 16519 mutations and the 16182C, 16183C, and 16194C). One

hundred and forty-nine additional complete sequences from the literature were employed for tree reconstruction and age estimation (Ingman et al., 2000; Mishmar et al., 2003; Tanaka et al., 2004; Macaulay et al., 2005; Starikovskaya et al., 2005; Kong et al., 2003, 2006; Derenko et al., 2007; Ingman and Gyllensten, 2007; Tamm et al., 2007; Bilal et al., 2008; Hartmann et al., 2008; Soares et al., 2008; Chandrasekar et al., 2009; Zhao et al., 2009).

### Principal-component analysis and multidimensional scaling plot

Principal-component (PC) analysis was performed using mtDNA haplogroup frequencies as input vectors by SPSS15.0 software (SPSS). Nonparametric multidimensional scaling (MDS) analysis based on  $F_{ST}$  statistics calculated from HVS-I sequences was also performed using SPSS15.0 software (SPSS) to visualize relationships among Tibetan populations and other Asian populations around. Population data of mtDNA diversity in East (Qian et al., 2001; Yao et al., 2002; Yao and Zhang, 2002; Wen et al., 2004a,b, 2005; Li et al., 2007), North (Horai et al., 1996; Kong et al., 2003; Derenko et al., 2007; Volodko et al., 2008), Central (Yao et al., 2000, 2004; Comas et al., 2004; Quintana-Murci et al., 2004; Heyer et al., 2009), South (Cordaux et al., 2003; Fornarino et al., 2009), and Southeast (Fucharoen et al., 2001; Hill et al., 2006, 2007) Asia were retrieved from the literature and included in our comparative analysis.

## RESULTS

### MtDNA haplogroup profiles

Detailed sequence variations and haplogroup assignments of 562 mtDNAs from 11 populations in this study are presented in Supporting Information Table S3. A total of 48 haplogroups or paragroups (unclassified lineages within a clade marked with an asterisk [\*]) were observed in our samples, all within the three principal non-African macrohaplogroups: M, N, and R (under N). Table 1 presents the haplogroup frequencies of the studied populations. The majority of the mtDNA lineages belong to eastern Eurasian groups. Only 2.4% mtDNAs can be traced for their origins to western or southern Eurasia, including one haplogroup J1b1 sample, one U2,



TABLE 1. Haplogroup frequencies of mtDNA of the 11 indigenous populations on the Tibetan plateau

Haplogroup	Tibetan										
	Yushu (44)	Nagqu (58)	Chamdo (61)	Ngari (46)	Nyingchi (53)	Shannan (56)	Shigatse (59)	Lhasa (59)	Garze (55)	Monba (51)	Lhoba (20)
A4	13.6	6.9	1.6	8.7	9.4	5.4	5.1	3.4	10.9	23.5	5.0
A7	...	...	...	...	...	...	...	...	...	...	10.0
A10	4.5	1.7	1.6	10.9	3.8	16.1	13.6	5.1	1.8	...	...
B*	...	...	1.6	...	...	...	...	...	...	...	...
B4*	4.5	...	3.3	...	...	3.6	...	...	3.6	2.0	...
B4a	4.5	1.7	...	2.2	...	...	...	...	...	2.0	...
B5b	...	...	1.6	...	...	1.8	...	...	3.6	...	...
C*	...	...	...	...	...	...	...	...	...	3.9	...
C4	4.5	...	1.6	2.2	3.8	7.1	6.8	...	...	...	...
C4d	2.3	3.4	1.6	2.2	1.9	1.8	...	...	5.5	...	5.0
C5	...	...	1.6	...	...	...	...	...	...	...	...
D2b	...	...	1.6	...	1.9	...	...	...	...	...	...
D4	15.9	6.90	6.56	4.35	15.09	14.29	6.78	10.17	12.73	1.96	15
D4j1	...	3.45	1.64	4.35	3.77	3.57	1.69	...	3.64	1.96	...
D5	...	3.4	3.3	...	...	3.6	...	1.7	...	2.0	...
D5a2	4.5	3.4	1.6	4.3	3.8	...	...	1.7	...	3.9	15.0
F*	2.3	...	...	...	...	...	...	...	...	...	...
F1	...	1.7	1.6	2.2	1.9	...	...	1.7	...	3.9	...
F1a	2.3	...	...	...	...	...	...	...	...	...	...
F1b	...	8.6	8.2	2.2	7.5	3.6	13.6	15.3	7.3	13.7	5.0
F2a	...	...	4.9	...	...	...	1.7	...	1.8	...	...
G*	2.3	6.9	3.3	...	3.8	...	1.7	1.7	1.8	...	5.0
G1a1	...	...	...	...	...	...	1.7	1.7	...	...	...
G2	2.3	1.7	3.3	4.3	...	1.8	1.7	6.8	5.5	5.9	...
G3	2.3	6.9	4.9	2.2	1.9	...	...	3.4	9.1	5.9	10.0
J1b1	...	...	...	...	...	...	1.7	...	...	...	...
M*	6.8	...	...	...	1.9	...	1.7	1.7	1.8	5.9	10.0
M7b2	2.3	...	...	...	...	...	...	...	...	...	...
M7c	...	...	...	...	...	...	...	...	1.8	...	...
M10	2.3	...	1.6	2.2	1.9	...	...	...	...	...	...
M11a	...	...	1.6	...	1.9	...	...	...	...	...	...
M13*	...	1.7	...	...	...	...	...	...	1.8	2.0	...
M13a	12.3	1.7	1.6	...	...	...	...	...	7.3	...	...
M13b	...	3.4	1.6	4.3	3.8	1.8	1.7	...	3.6	3.9	5.0
M62	...	5.2	1.6	...	1.9	5.4	1.7	3.4	...	...	...
M8a	...	...	1.6	...	...	...	...	1.7	3.6	...	...
M9a	2.3	6.9	13.1	23.9	13.2	8.9	25.4	25.4	1.8	11.8	...
M9d	6.8	6.9	13.1	10.9	13.2	16.1	6.8	8.5	1.8	3.9	10.0
M9e	...	8.6	...	...	...	...	...	...	...	...	...
N*	...	1.7	...	...	...	1.8	...	...	...	...	...
N9a	...	...	...	...	1.9	...	...	...	...	...	...
R*	6.8	...	...	2.2	1.9	...	...	...	...	...	...
R9b	...	1.7	...	...	...	...	...	...	...	...	...
T1	...	...	...	4.3	...	...	...	3.6	...	...	...
U2	...	...	1.6	...	...	...	...	...	...	...	...
U7	...	3.4	...	...	...	...	3.4	1.7	...	2.0	...
U5	2.3	...	...	...	...	...	...	...	...	...	...
Z	...	1.7	3.3	...	...	3.6	1.7	5.1	1.8	...	...

one U5, six U7, and four T1 samples. This pattern is consistent with the notion that Himalayas served as a barrier to gene flow (Gayden et al., 2007, 2009). Among the eastern Eurasian component, 67.6% belongs to macrohaplogroup M and its derived haplogroups (such as C, D4, D5, G, M\*, M7, M8, M9, M10, M11, M13, and Z), and 30.1% belongs to macrohaplogroup N and its nested haplogroups (A, B, F, N9a, R9b, N\*, and R\*). Within macrohaplogroup M, haplogroup D and M9 are the most common lineages, accounting for 20.3% and 13.6% of all samples, respectively. The most prevalent haplogroups within macrohaplogroup N, haplogroup A and F represented 8.6% and 11.5% of the studied lineages. Although the Tibetan Plateau is located in southwestern part of China, the majority of maternal gene pool on the plateau showed a strong similarity with north Asian populations,

which was evident by the high proportion of north Asian-prevalent haplogroups (Tanaka et al., 2004; Derenko et al., 2003, 2007), such as haplogroups A, C, D4, D5, F1b, M9, and G. It is also worth noting that haplogroup B, which is one of the most common lineages in southern and eastern Asian populations, exhibited low frequency at the southern part of the plateau, accounting for only 5.4% in Shannan Tibetans, 4.0% in Monba, and it was not found in Nyingchi, Shigatse, and Lhasa Tibetans, nor in Lhoba.

### Population summary statistics

Internal population diversity indices and results of Tajima's *D* and Fu's *F<sub>s</sub>* neutrality tests are presented in Table 2. All the studied populations exhibited high and

TABLE 2. Diversity indices and results of neutrality tests of the 11 indigenous populations on the Tibetan Plateau, based on 440-bp-length sequences (from np 16024–16463) of HVS-I region

Population	$n^a$	$H^b$	$K^c$	$S^d$	$\Pi^e$ (SE)	$\theta_k$ (95% CI)	Tajima' $D(P)^f$	Fu's $F_s(P)^f$
Lhoba	20	0.9842 (0.0205)	17	34	6.884 (3.776)	50.68 (18.75–149.75)	-1.11 (0.125)	-7.86
Monba	51	0.9702 (0.0125)	33	45	6.138 (2.968)	39.38 (22.64–69.37)	-1.32 (0.072)	-20.15
Tibetan_Ngari	46	0.9778 (0.0125)	35	51	6.594 (3.172)	65.06 (34.90–125.38)	-1.51	-24.69
Tibetan_Chamdo	61	0.9896 (0.0065)	51	68	6.411 (3.783)	143.83 (78.19–276.80)	-1.91	-25.17
Tibetan_Garze	55	0.9886 (0.0059)	42	50	6.194 (2.988)	79.44 (44.62–145.71)	-1.48	-25.22
Tibetan_Lhasa	59	0.9813 (0.0114)	49	63	6.145 (2.964)	133.26 (72.24–257.01)	-1.87	-25.23
Tibetan_Nyingchi	53	0.9855 (0.0080)	41	56	5.901 (2.863)	81.25 (44.92–151.86)	-1.80	-25.30
Tibetan_Nagqu	58	0.9897 (0.0056)	45	61	6.635 (3.178)	90.36 (51.05–164.96)	-1.70	-25.12
Tibetan_Yushu	44	0.9937 (0.0067)	39	60	6.937 (3.324)	160.77 (73.29–382.20)	-1.76	-25.08
Tibetan_Shigatse	59	0.9468 (0.0219)	38	59	6.338 (3.048)	45.06 (26.86–76.43)	-1.71	-24.60
Tibetan_Shannan	56	0.9812 (0.0089)	41	53	6.135 (2.962)	67.46 (38.68–120.46)	-1.60	-25.24

<sup>a</sup> Sample size.

<sup>b</sup> Haplotype diversity.

<sup>c</sup> Number of different haplotypes.

<sup>d</sup> Number of segregating sites.

<sup>e</sup> Average number of pairwise differences.

<sup>f</sup> All  $P$  values are  $<0.05$  except where noted.

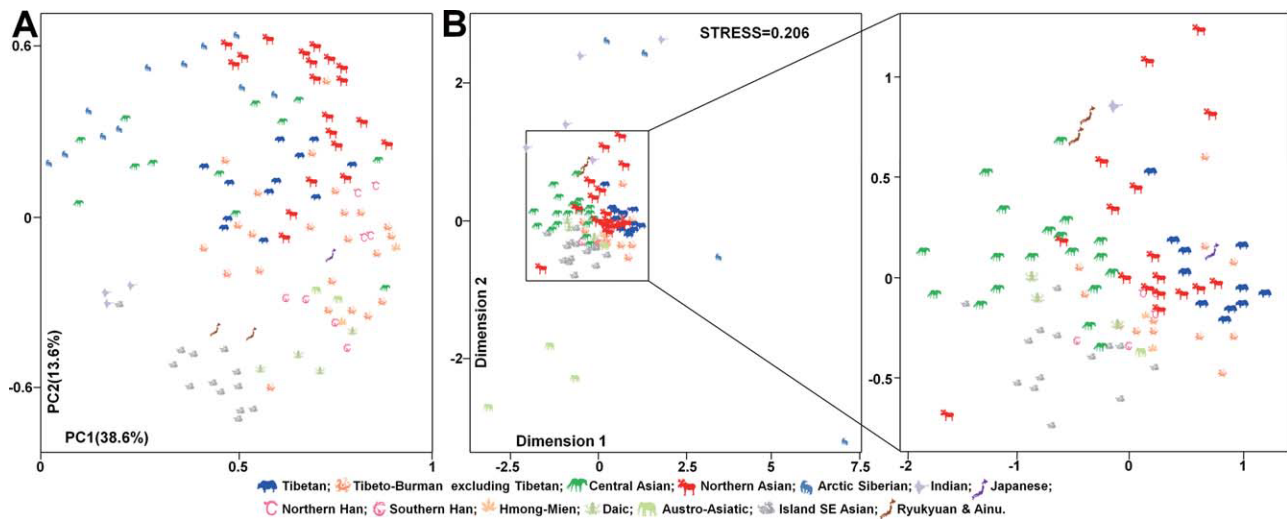


Fig. 2. Principal component plot (A) and multidimensional scaling (B) of 112 populations residing in eastern Eurasia. Populations are grouped with either geographical distribution or linguistic classification as shown in the plot. [Color figure can be found in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com)]

similar diversity levels. The haplotype diversity ( $H$ ) ranged from 0.947 to 0.994, and the mean number of pairwise differences observed ( $\pi$ ) ranged from 5.90 to 6.94. Most population samples yielded significant negative values for both Tajima's  $D$  and Fu's  $F_s$  neutrality tests, suggesting historical population expansion. Lhoba and Monba population showed significant negative Fu's  $F_s$  value, but the results of Tajima's  $D$  test were not significantly different from zero. This contradiction perhaps resulted from mutation-rate heterogeneity of the HVS-I region, which can influence the signature of population expansion in Tajima's  $D$  test as previously reported (Tanaka et al., 2004; Derenko et al., 2003, 2007).

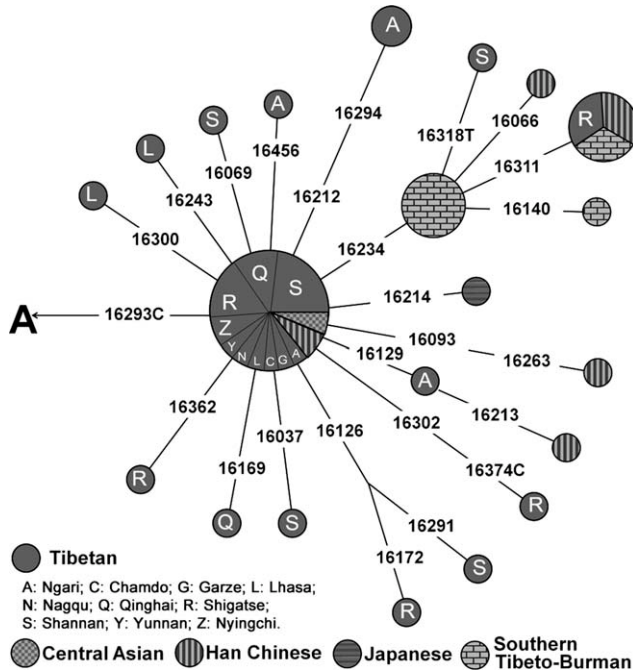
### PC analysis and MDS plot

Samples from the 11 populations in current study and 101 populations residing in eastern Eurasia were included in principal component analysis. All of these 112 population samples were plotted using the first two PCs (Fig. 2A), which accounted for 38.6% and 13.6% of

the total variation, respectively. These first two PCs mainly reflected geographic distributions of the populations. Tibetan populations clustered closely with northern Asian populations, northern Han Chinese, and other Tibeto-Burman populations residing in Nepal and India. This close relationship may indicate a common ancestor of their maternal gene pools. In contrast, Tibeto-Burman populations from southern China scattered widely and part of them clustered closely to populations from southern East Asia, reflecting strong influences of southern East Asian inhabitants on maternal diversity of southern Tibeto-Burman populations as we previously reported (Wen et al., 2004b).

The strong affinity of Tibetans and northern Asian populations was also reflected in MDS plot (Fig. 2B). Central Asian populations were segregated from the Tibetans clearly by the northern Asian populations in the first dimension of the MDS plot. Southern Tibeto-Burman populations distributed closely in the plot with southern Han Chinese, Daic populations, and Southeast Asian populations.





**Fig. 4.** Median-joining network of the newly identified subhaplogroup A10, based on HVS-I sequences between the region np 16024–16463.

mutations (at np 1005, 6755, 8843), named as A10a; one sequence from Shannan Tibetans and the only one published A10 full sequence from the Naxi population (neighboring the Tibetans) living in Yunnan China (Hartmann et al., 2008) had a control-region substitution at site 16234, assigned as A10b. It is noteworthy that sublineage A10b has been found outside the Tibetan Plateau (mostly in Tibeto-Burman speaking populations residing in Yunnan China), suggesting that these people migrated southward from the plateau. This observation is concordant with our previous conclusion about the genetic structure of Tibeto-Burman populations (Wen et al., 2004b). The Tibetan A4 sequences represented as novel haplotypes within the subhaplogroup A4 shared no coding-region mutation with any previously published A4 sequences (16362 is the only marker defining A4).

Network analysis of HVS-I sequences of lineage A10 revealed a star-like pattern and thus showed a signal of population expansion on the plateau (Fig. 4), which was also confirmed by unimodal mismatch distribution in this subhaplogroup (data not shown). The A10 lineage expansion time was estimated to  $15,100 \pm 4,700$  YBP. Importantly, database searching also identified a similar HVS-I motif of 16242-16293C-16319 in Mongolia (Kolman et al., 1996) and southern Siberia (Derenko et al., 2003) populations. However, previously published complete sequences (Starikovskaya et al., 2005; Derenko et al., 2007) grouped these samples to northern Asian specific lineage A8, and thus they were excluded in Network analysis.

### Haplogroup M62

Haplogroup M62 was first reported as a novel haplogroup in northeast India (Chandrasekar et al., 2009). However, recent published 13 complete sequences of M62 from Tibet indicated that it is Tibetan specific

(Zhao et al., 2009). In this study, we confirmed this specificity by intensively coding region mutation screening. Three M62 samples were also selected to be complete sequenced to update the M62 phylogeny, which was illustrated in Figure 5. We revised the classification of haplogroup M62, as having three subclade: M62a, characterized by one control-region (203) transition; M62b, characterized by three transitions (3693, 6305, and 7364) and one transversion (187T); and M62c, characterized by one back mutation at np 204. Two Indian M62 sequences shared five transitions (146, 310, 4763, 9935, and 16147) with our sample from Shigatse (RKZ5474), nesting in M62c branch. Actually, these Indian sequences were also belonged to Tibeto-Burman speaking populations, indicating their origin in the Tibetan Plateau.

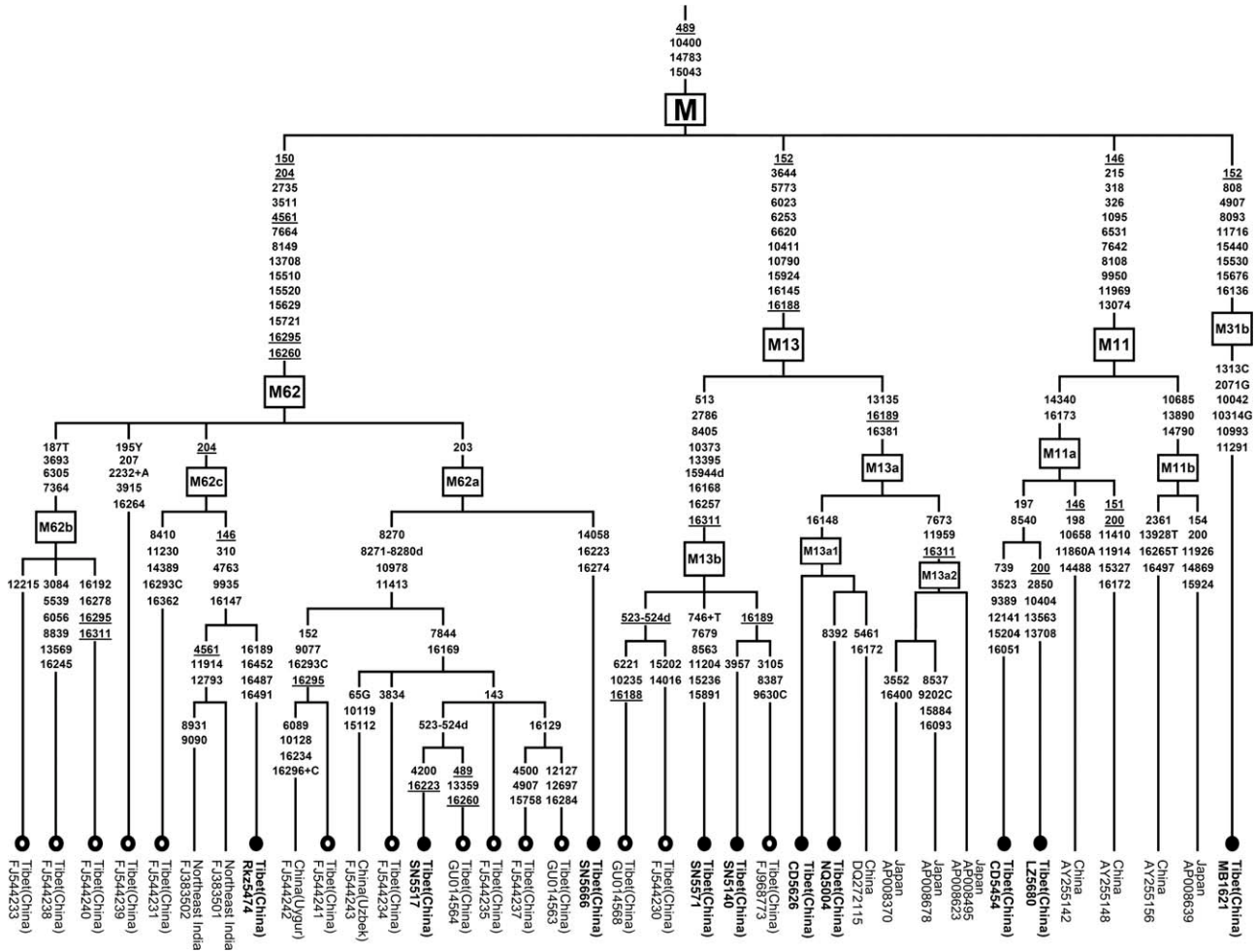
### Subhaplogroup C4d

Haplogroup C is another common lineage that is widespread in East Asia and Siberia and is one of the founder lineages among Native Americans (Torroni et al., 1993). The entire C haplogroup is characterized by the HVS-I motif 16223-16327 and five mutations in the coding region (3552, 9545, 11914, 13263, and 14318). This haplogroup was detected in populations of the Tibetan Plateau with considerable frequencies (average 4.7%). Almost 40% (11/28) of our haplogroup C samples harbored a specific HVS-I motif 16093-16298-16327 and missed one of the characteristic mutations at np 16223. We sequenced two complete mtDNA genomes (LZ5332, SN5515) belonging to this type and performed phylogenetic analysis. The results demonstrated that these two sequences clustered closest with sublineage C4, but lacked an Adenine (A) insertion at np 2232, which is one of the four coding-region mutations (2232A ins., 6026, 11969, 15204) defining subhaplogroup C4 Starikovskaya et al., 2005). Additional screening at np 2232 in all these eleven samples bearing HVS-I motif 16093-16298-16327 confirmed that none of them had 2232A insertion. Searching for homologous HVS-I motifs in databases resulted only one identical subject from Qinghai Tibetans (Wen et al., 2004b), thus suggesting that this haplotype may be Tibetan specific. We then reconstructed the phylogeny of subhaplogroup C4 with our newly sequenced samples (LZ5332, SN5515, and SN5668) and all previously published C4 mtDNA genomes (Fig. 6). Subhaplogroup C4 was redefined here and was characterized by only three coding-region mutations (6026, 11969, 15204) and two control-region mutations (249 and 16327). We referred to this Tibetan-specific lineage as subhaplogroup C4d. TMRCA of subhaplogroup C4d was calculated to be  $20,600 \pm 6,800$  years ago. It should be noted that two complete sequences share three additional mutations (7100, 12780, and 15236) in coding region, also indicating a significant divergence within the Tibetan-specific C4d branch.

### Subhaplogroup M13b

Tibetan distinctiveness has also been found in haplogroup M13. This haplogroup is presented in East Asian populations at very low frequency and has been found sporadically in northern Asia (Fedorova et al., 2003; Pakendorf et al., 2003; Derenko et al., 2007). On the plateau, the frequency of haplogroup M13 is remarkable, accounting for 4.3% (24/562) samples. Moreover, 58.3% (14/24) of these M13 samples harbored a distinguishing





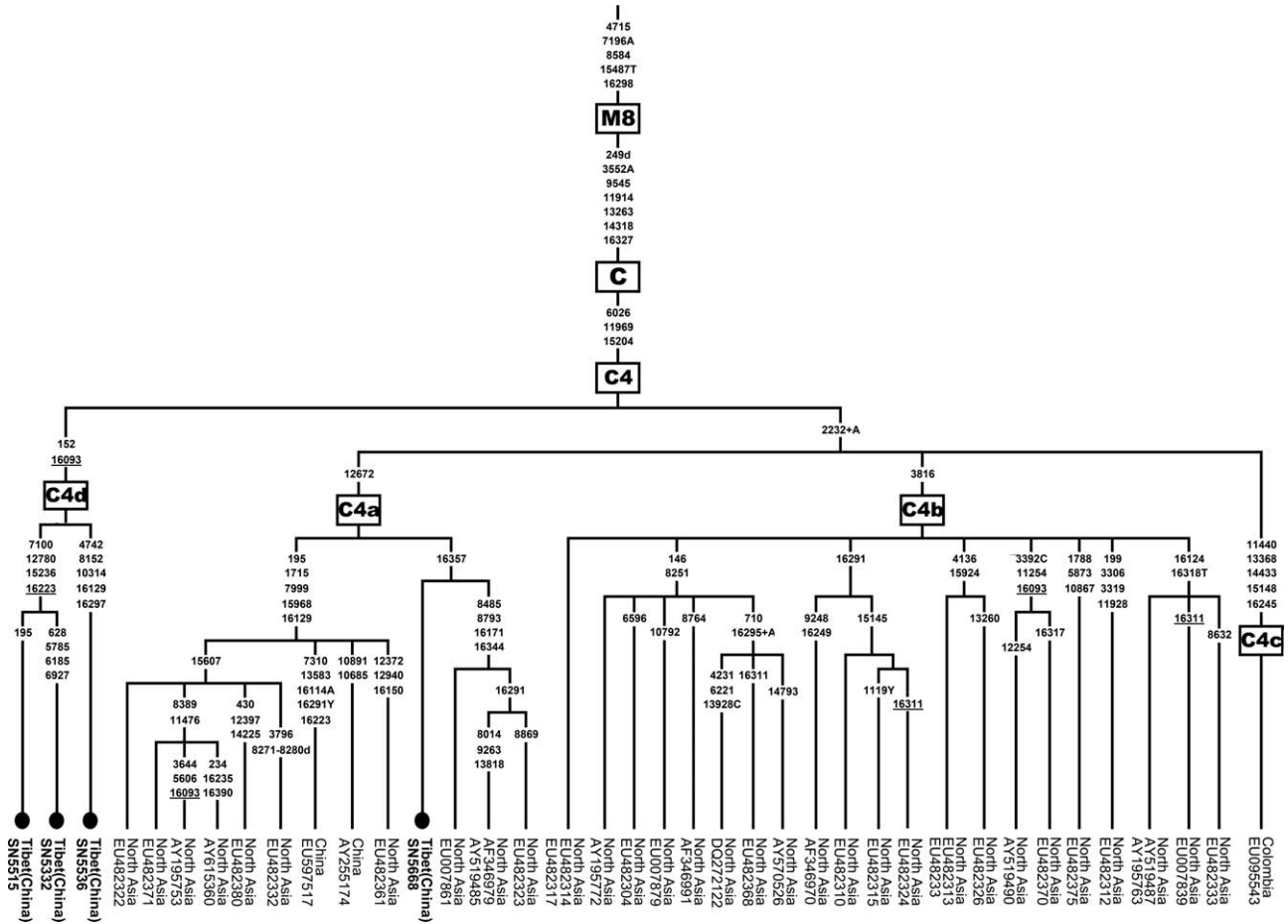
**Fig. 5.** The phylogenetic tree of complete mtDNA sequences of haplogroup M11, M13, and M62. The tree is rooted in macrohaplogroup M. For additional information, see the Figure 3 legend.

HVS-I motif: 16145-16168-16223-16257-16311. Extensive literature search revealed that this specific lineage was only observed in populations of Tibeto-Burman linguistic branch residing closely to Tibetan Plateau, e.g., one Tibetan (Yao and Zhang, 2002), one Bai and two Pumi (Wen et al., 2004b) from Yunnan of China, one Lisu and one White Karen individual from northern Thailand (Oota et al., 2001) (Supporting Information Table S4). Thus we defined this special mtDNA as M13b. We sequenced four complete Tibetan mtDNA genomes and compared them with all published M13 complete sequences (Fig. 5). A Mongolian sample (MG50) (Kong et al., 2006) nested with two Tibetan samples (CD5626, NQ5004) and formed a subclade, M13a1, characterized by control-region mutation at np 16148. The Japanese mtDNAs clustered into another branch, M13a2, characterized by two coding-region mutations (7673 and 11959). It was noteworthy that the two Tibetan M13b mtDNAs (SN5140, SN5571) differed from the rest of haplogroup M13 by six coding-region and four control-region transitions. The divergence time of M13a and M13b was calculated as  $21,100 \pm 6,100$  years. With the exclusive distribution of subhaplogroup M13b in Tibeto-Burman populations, this deep divergence time may indicate the late Pleistocene modern human settlement and long-time isolation.

### Other MtDNA lineages presented on the Tibetan plateau Haplogroup M9

One major component of mtDNA pool on the plateau is represented by haplogroup M9, which distinguished the Tibetans from other East Asian populations. As previously reported (Kivisild et al., 2002; Yao et al., 2002; Soares et al., 2008), haplogroup M9 encompassed two subclades: E and M9a. While subhaplogroup E is restrictedly distributed in Island Southeast Asia (ISEA) and Taiwan (Trejaut et al., 2005; Hill et al., 2007; Soares et al., 2008), lineage M9a was widely presented in mainland East Asia and reached its greatest frequency and diversity in Tibet (Torroni et al., 1994; Tanaka et al., 2004). In this study, the average frequency of haplogroup M9a on the Tibetan Plateau was 21.6% with the highest frequencies in the ethnic Tibetans from Ngari (34.8%), Shigatse (32.2%), and Lhasa (32.8%). Sublineage M9a has been detected in central (Yao et al., 2004), northern (Derenko et al., 2007), and eastern (Tanaka et al., 2004; Soares et al., 2008) Asian populations, but all in low frequencies (<5%). In addition, it has been reported that the deepest branches of haplogroup M9 (pre-M9a) were found in Indochina, Mainland China, Taiwan, and the





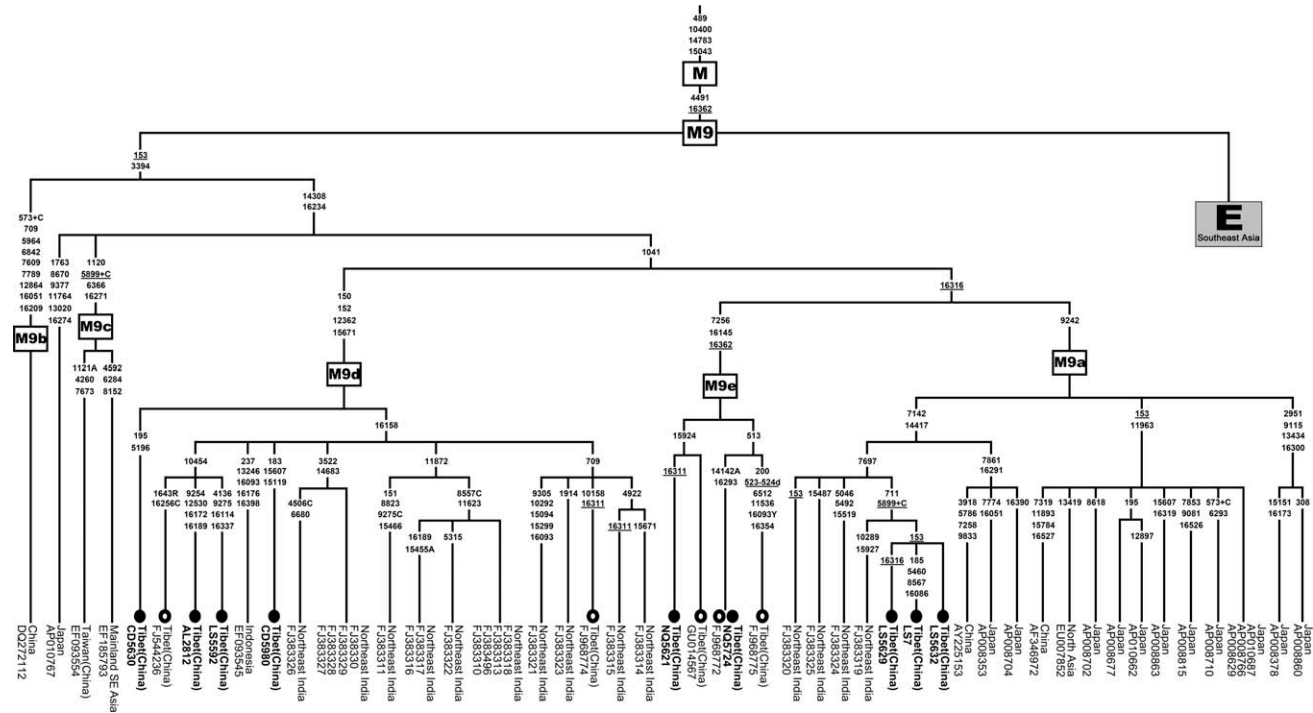
**Fig. 6.** The phylogenetic tree of complete mtDNA sequences of subhaplogroup C4. The tree is rooted in macrohaplogroup M. For additional information, see the Figure 3 legend.

next deepest in ISEA (Soares et al., 2008). On the plateau, M9 haplogroup is represented as four distinct HVS-I motif types: 16223-16234-16362 (pre-M9a), 16223-16234-16316-16362 (M9a), 16158-16223-16234-16362 (M9d), and 16145-16223-16234. To further assess the lineage M9 variations found in mitochondrial gene pool of the Tibetan Plateau, we selected 10 samples of all the above four different motifs and sequenced their complete mtDNA genomes. Combining all published haplogroup M9 (excluding sublineage E) mtDNA genomes (Tanaka et al., 2004; Kong et al., 2006; Bilal et al., 2008; Soares et al., 2008; Chandrasekar et al., 2009), and our nine newly collected samples, we reconstructed a tree of 56 complete sequences (Fig. 7). According to this updated phylogenetic tree, we defined one new M9 subclade and its diagnostic coding SNPs as: M9e (7256C). Interestingly, two sequences (CD5630, LS5629) harboring 16223-16234-16362 HVS-I motif, which have been designated as pre-M9a previously, fall into subclades M9d and M9a, respectively. Additional screening of mutations at np 9242, 12362, and 7256 in these pre-M9a samples revealed that they all belong to either M9a or M9d subclades. Notably we found that the only published M9d sequence (EF093545) from Southeast Asia nested in Tibeto-Burman samples, and the M9d frequency was much higher on the Tibetan Plateau (8.9%) than in

Southeast Asia. Thus this M9d sequence may indicate a recent gene flow from Tibet to Southeast Asia.

### Haplogroup D2b and D4j

The distribution of subhaplogroup D2b (“D2a” in (Derenko et al., 2007)) in Asian populations indicated a southern Siberian rather than Beringian origin of haplogroup D2 lineages. In our samples from the Tibetan Plateau, we found three mtDNA sequences (LZ1572, LZ1600, and CD5479) bearing similar haplogroup D2 HVS-I motif (16129-16223-16271-16362). Moreover, our results from complete mtDNA sequencing and selected variation checking revealed that two of these sequences (LZ1600 and CD5479) were similar to the southern Siberian-specific D2b sublineage but lacked the substitution at np 5004, which is one of the four mutations (at np 195, 5004, 9181, and 16092) defining subhaplogroup D2b as previously reported (Derenko et al., 2007). The updated phylogeny of D2b was illustrated in Figure 8. These two Tibetan sequences showed strong affinity with the southern Siberian D2b sequences. The other sequence (LZ1572), bearing transitions at np 5262 and 11969, grouped into D4j1 cluster (Fig. 8), which was newly identified in Tibeto-Burman populations from Northeast India (Chandrasekar et al., 2009). Screening



**Fig. 7.** The phylogenetic tree of complete mtDNA sequences of haplogroup M9. The sequences of subhaplogroup E are not presented in the phylogeny. The tree is rooted in macrohaplogroup M. For additional information, see the Figure 3 legend.

at np 5262 revealed 16% (12/75) of haplogroup D4 samples on the plateau belonged to D4j1.

### Haplogroup M11 and M31b

We have also completely sequenced three M\* genomes that cannot be further classified. Two sequences (LZ5454 and CD5680) fell into haplogroup M11 distinguished by seven coding-region mutations (1095, 6531, 7642, 8108, 9950, 11969, and 13074) and four mutations in HVS-II region (146, 215, 318, and 326). It should be noted that these two sequences showed deep divergence with each other, pointing to a remarkable divergence on the plateau. Another M\* sample (MB1621) from the Monba population belonged to sublineage Tibeto-Burman populations from Northeast India (Chandrasekar et al., 2009). Screening at np.

### Time estimate

The coalescence time and variation computed from representative lineages on the plateau (A10, C4d, M62, M13, M13b, M11, M9a, M9d, M9e, and D2b) were given in Table 3. The old age and the restricted geographic distribution of haplogroup M62 on the Tibetan Plateau indicated that this haplogroup may have evolved in situ on the plateau before LGM, which have also been suggested by other study recently (Zhao et al., 2009). Other haplogroups such as A10, C4d, and M13b also showed strong association with the Tibetans. These lineages outside the plateau in very low frequency are either in the Tibeto-Burman ethnic groups which shared common ancestry with the Tibetans or in the populations living with the Tibetans (Supporting Information Table S3). The coalescence time estimates of the A10 and C4d subclades were also around 20KYA. However, the synonymous mutation

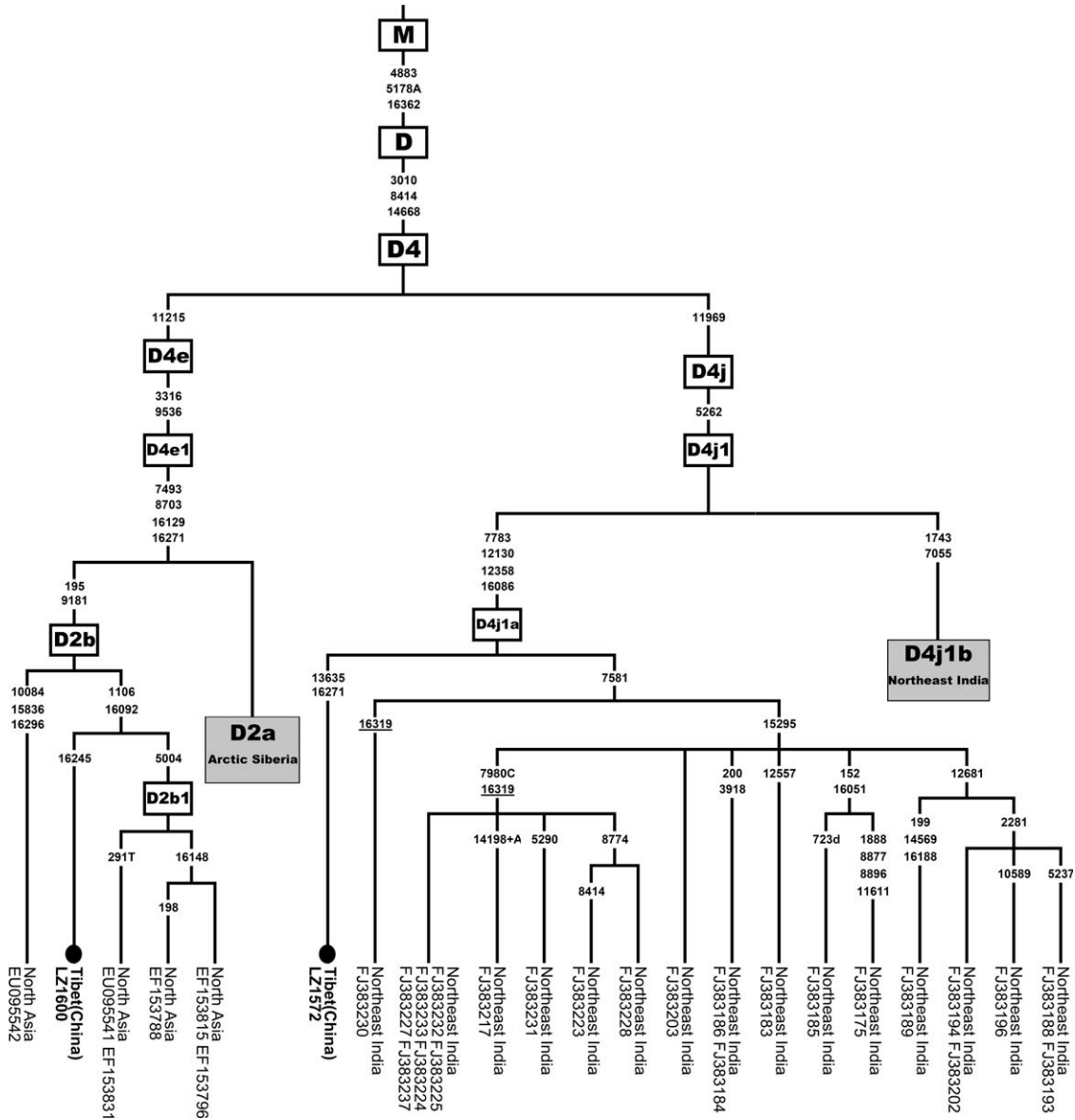
methods issued more recent estimates, indicating the limitations of this method (Soares et al., 2009). Although most methods indicated the oldness of A10, C4d, and M13b, we cannot yet rule out the possibility of the post-LGM appearances of these lineages on the plateau, therefore, these lineages can only be regarded as putative pre-LGM lineages.

The ages of the three M9 subclades (M9a, M9d, and M9e) fell into early Holocene (about 12KYA), showing a remarkable gap with the ages of the putative pre-LGM lineages (around 20KYA). The Tibetan M9a samples all harbored three additional coding region mutations (7142, 7697, and 14417), indicating the recent appearance of this lineage in Tibetan populations. We also estimated the age of the D2b cluster as about 10KYA. The Tibetan samples of D2b nested in the Siberian prevalent lineages, indicating recent gene flows between the two regions.

## DISCUSSION

### Common ancestry of the Northern Asians and the Tibetan populations

Analyses of 562 mtDNA sequences from 11 Tibeto-Burman populations residing in different regions of the Tibetan Plateau showed that the maternal variation on plateau was largely contributed by northern Asian-prevalent haplogroups, thus testifying a common maternal ancestry of the Tibetan populations and northern Asian populations (Torroni et al., 1994; Gayden et al., 2009). This notion was confirmed by both PC analysis and MDA plots, where the Tibetan populations clustered closely to the northern Asian groups and separated from central and southern Asian populations. However, unlike the northern Asian populations, the Tibetan populations contents very low frequencies of western Eurasian



**Fig. 8.** The phylogenetic tree of complete mtDNA sequences of subhaplogroup D2 and D4j. The tree is rooted in macrohaplogroup M. For additional information, see the Figure 3 legend.

genetic components, indicating that the Tibetan Plateau is more like a “genetic capsule” area. Moreover, extremely low frequencies of the South Asian lineages on the plateau reflected the strong “barrier effect” of the Himalayas between the Indian subcontinent and the Tibetan Plateau (Gayden et al., 2007; Kang et al., 2010).

**Evidences of the pre-LGM human activity on the plateau**

Archeological sites of more than 20,000 years old (Huang, 1994; Zhang and Li, 2002; Yuan et al., 2007) supported a pre-LGM human activity on the Tibetan Plateau. Notably, some of these pre-LGM Paleolithic sites were found in the northern part of the plateau, where there are very few current inhabitants living with harsh climate, indicating that most regions of the plateau was inhabited in the pre-LGM period. During the LGM, snow accumulated in the Tibetan Plateau and

developed into greater glacier and permafrost (Lehmkuhl and Haselein, 2000; Zheng et al., 2003), which might have “closed” most of the plateau, resulting in a large decrease of the local human population.

Recently, Zhao et al. (2009) claimed that an infrequent haplogroup (M16) in the Tibetan populations may represent the genetic relics of the Late Paleolithic inhabitants on the plateau. In our study, we also found some old lineages (M62, A10, and C4d) which may be the remains of pre-LGM inhabitants, providing evidences for LGM survivals. However, the age of a single haplogroup may not be the age of a population demographic event. If the people who colonized the area carried part of the original diversity not just one founder, the TMRCA of a haplogroup can be much older than the colonization event, and this haplogroup can in some cases have reached much lower frequency in the source population because of drift. Conversely if the founding group had a small effective population size, the TMRCA of a haplogroup can be much



TABLE 3. Age estimations of some haplogroups found in Tibetan Plateau based on different calibration rates

Haplogroup	n	Mishmar rate <sup>a</sup>		Modified Mishmar rate <sup>b</sup>		Modified Kivisild rate <sup>c</sup>		Soares synonymous rate <sup>d</sup>		Soares complete genome rate <sup>e</sup>	
		$\rho \pm \sigma$	T(ky)	$\rho \pm \sigma$	T(ky)	$\rho \pm \sigma$	T(ky)	$\rho \pm \sigma$	T(ky)	$\rho \pm \sigma$	T(ky)
M62	18	4.75 ± 0.88	24.42 ± 4.54	4.75 ± 0.88	21.90 ± 4.07	2.94 ± 0.89	22.53 ± 6.81	2.94 ± 0.89	23.21 ± 7.02	8.61 ± 1.06	23.67 ± 6.06
M11	6	5.33 ± 1.20	27.41 ± 6.18	5.33 ± 1.20	24.59 ± 5.54	2.17 ± 0.73	16.58 ± 5.56	2.17 ± 0.73	17.08 ± 5.73	8.17 ± 1.48	22.38 ± 8.43
C4d	3	4.33 ± 1.45	22.27 ± 7.47	4.33 ± 1.45	19.98 ± 6.70	2.33 ± 0.88	17.85 ± 6.75	2.33 ± 0.88	18.40 ± 6.95	8.00 ± 2.05	21.89 ± 11.63
A10	8	5.00 ± 1.15	25.70 ± 5.89	5.00 ± 1.15	23.05 ± 5.28	1.63 ± 0.67	12.43 ± 5.15	1.63 ± 0.67	12.81 ± 5.31	6.88 ± 1.28	18.67 ± 7.16
M13	12	5.00 ± 0.82	25.7 ± 4.20	5.00 ± 0.82	23.05 ± 3.76	1.75 ± 0.61	13.39 ± 4.64	1.75 ± 0.61	13.80 ± 4.78	9.00 ± 1.15	24.81 ± 6.61
M13b	5	2.80 ± 0.85	14.39 ± 4.36	4.33 ± 1.45	12.91 ± 3.91	1.00 ± 0.60	7.65 ± 4.59	1.00 ± 0.60	7.88 ± 4.73	4.00 ± 1.06	10.61 ± 5.69
M9a	19	3.11 ± 0.70	15.96 ± 3.60	3.11 ± 0.70	14.32 ± 3.23	1.32 ± 0.53	10.07 ± 4.09	1.32 ± 0.53	10.37 ± 4.21	4.74 ± 0.83	12.64 ± 8.50
M9d	7	2.00 ± 0.83	10.28 ± 4.28	2.00 ± 0.83	9.22 ± 3.84	0.57 ± 0.57	4.37 ± 4.37	0.57 ± 0.57	4.51 ± 4.51	4.57 ± 1.37	12.18 ± 7.42
M9e	5	1.20 ± 0.85	6.17 ± 4.36	1.20 ± 0.85	5.53 ± 3.91	0.40 ± 0.28	3.06 ± 2.16	0.40 ± 0.28	3.15 ± 2.23	3.20 ± 0.94	8.43 ± 4.97
D2b	7	1.86 ± 1.22	9.55 ± 6.27	1.86 ± 1.22	8.56 ± 5.63	0.71 ± 0.71	5.46 ± 5.46	0.71 ± 0.71	5.46 ± 5.46	3.86 ± 1.21	10.22 ± 6.49

Coalescence times were estimated based on rho( $\rho$ ) statistics with different mutation rates: Coding region substitutions.

<sup>a</sup>Mishmar rate,

<sup>b</sup>coding region substitutions Modified Mishmar rate,

<sup>c</sup>coding region synonymous transitions Modified Kivisild rate,

<sup>d</sup>coding region synonymous substitutions Soares synonymous rate, and

<sup>e</sup>complete genome substitution Soares rate. n indicates number of sequences,  $\sigma$  indicates number of sequences,  $\sigma$  indicates number of sequences,  $\sigma$  indicates number of sequences, and CI indicates confidence interval.

younger than the colonization time. In this study, time estimates of several haplogroups resulted in similar old ages of around 22,000 years, making it less possible to be an over estimate of the colonization time. Giving the inhospitable environment of the Tibetan Plateau and the population decreasing during LGM, the effective population size on the plateau might be very small at least in part of the history; therefore, the colonization time of the plateau could be older than our estimates. This consequence mostly supports the pre-LGM colonization of the plateau suggested by archeology findings.

### Multiple origins of Tibetan populations

The exclusive distribution of mtDNA sublineages on the plateau enabled us to estimate the colonization time of the plateau. Some of these plateau-specific lineages (A10, C4d, and M62) seem to have evolved on the plateau for a long time, indicating that the first entry of modern human into the Tibetan Plateau might have happened before LGM deteriorated the plateau climate substantially (Aldenderfer and Yinong, 2004). In this respect, subclade A10 provided further information about the pre-LGM settlers. Subhaplogroup A10 showed the highest frequency and diversity in Shannan and Shigatse of south Tibet. Considering the present suitable environment of south Tibet, especially that of Shannan, (Aldenderfer and Yinong, 2004), the warm valleys in south Tibet might have been the refugium for the pre-LGM settlers during LGM. In addition, this haplogroup showed a signal of population expansion, dating back to ~15 KYA, as a consequence of environment change after the LGM period.

Phylogeographic analysis of another major haplogroup on the plateau, M9, revealed a considerable genetic component of post-LGM migrants. However, the ancestral haplotypes of M9 was not found in the Tibetan populations, but in the populations of Southeast Asia, Japan, and coastal China (Jiangsu and Shandong province in China, author's unpublished data). Moreover, another lineage nested in haplogroup M9, subclade E, has a Pleistocene origination in east Sunda of Southeast Asia (Derenko et al., 2007; Soares et al., 2008). Combining the geographic distribution information of the M9 diversity, we supported the hypothesis that haplogroup M9 originated in Southeast Asia more than ~50,000 years ago and evolved into the subhaplogroup E and M9a-M9d, and M9a-M9d subsequently migrated northward probably during Late Pleistocene (Soares et al., 2008). Totally, the presence of only derived sublineages in the Tibetan Plateau indicates a counter-clockwise dispersal in mainland East Asia as previously proposed (Chaix et al., 2008). The high frequencies of sublineages M9a and M9d on the plateau may result from either demographic effect or selective effect (Gu et al., 2008), which requires further investigation.

The high frequency of Y chromosome polymorphic Alu insertion (YAP) in Tibet makes the origins of the Tibetans intriguing and controversial. Although multiple origins of the Tibetan people have been proposed by different studies in the last decade (Qian et al., 2000; Su et al., 2000), the sources and routes presented in such claims are highly debated (Qian et al., 2000; Su et al., 2000; Thangaraj et al., 2005; Shi et al., 2008). Central Asia has been considered as the one of the main sources of YAP (Qian et al., 2000; Su et al., 2000; Gayden et al., 2007). In this study, however, considering the extremely

low frequency of western or central Asian mtDNA haplogroups (2.3%), it is less likely that Central Asian is a major contributor, at least in the maternal aspect. Recently, Shi et al. (2008) demonstrated two independent Paleolithic dispersal events of modern human into East Asia of ~50 KYA (marked by Y haplogroup D-M174) and ~30 KYA (marked by Y haplogroup O-M175 and its derivatives) (Shi et al., 2005, 2008). Haplogroup D and O are both dominant Y haplogroups in Tibet, indicating multiple migrations into Tibet. The routes Shi et al. proposed of the two ancient human dispersals are consistent with our findings of pre- and post-LGM migrations to the Tibetan Plateau. This concurrence, however, needs to be investigated in our further study.

### Possible bias in time estimates

The genetic time estimates may be affected by several factors. The current statistic method for time estimate is still developing. A recent study showed that molecular dating with the rho statistic could produce biased results with large asymmetrical variances (Cox, 2008). Also demographic histories (such as bottlenecks, founder events and changes in effective population size) can particularly distort date estimates with the rho statistic (Cox, 2008; Endicott et al., 2009). Therefore, our conclusion may be revised in case that bias in time estimate exists, and Tibetan populations may have demographic histories different from our hypothesis. Furthermore, mutation rates to be used in mtDNA dating are yet to be determined. Researchers have demonstrated that Mishimar mutation rate always gave much older dates for East Asian populations (Endicott et al., 2009). However, we used multiple mutation rates to eliminate the bias, and the results are fairly consistent. Thus, we interpreted our data as pre- and post-LGM Tibetan plateau inhabitants with cautions. To make more solid conclusion, we need to generate sufficient Tibetan mtDNA complete sequences and analyze data with several different methods (Endicott and Ho, 2008; Ho and Endicott, 2008; Sores et al., 2009).

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