

A Western Eurasian Male Is Found in 2000-Year-Old Elite Xiongnu Cemetery in Northeast Mongolia

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ABSTRACT We analyzed mitochondrial DNA (mtDNA), Y-chromosome single nucleotide polymorphisms (Y-SNP), and autosomal short tandem repeats (STR) of three skeletons found in a 2,000-year-old Xiongnu elite cemetery in Duurlig Nars of Northeast Mongolia. This study is one of the first reports of the detailed genetic analysis of ancient human remains using the three types of genetic markers. The DNA analyses revealed that one subject was an ancient male skeleton with maternal U2e1 and paternal R1a1 haplogroups. This is the first genetic evidence that a male of distinctive Indo-European lineages (R1a1) was present in the Xiongnu of Mongolia. This might indicate an Indo-

European migration into Northeast Asia 2,000 years ago. Other specimens are a female with mtDNA haplogroup D4 and a male with Y-SNP haplogroup C3 and mtDNA haplogroup D4. Those haplogroups are common in Northeast Asia. There was no close kinship among them. The genetic evidence of U2e1 and R1a1 may help to clarify the migration patterns of Indo-Europeans and ancient East-West contacts of the Xiongnu Empire. Artifacts in the tombs suggested that the Xiongnu had a system of the social stratification. The West Eurasian male might show the racial tolerance of the Xiongnu Empire and some insight into the Xiongnu society. *Am J Phys Anthropol* 000:000–000, 2010. ©2010 Wiley-Liss, Inc.

When the Pontic-Caspian Steppe in the West of South Siberia was dominated by the Scythians, a branch of Iranians, the eastern portion of South Siberia was ruled by scattered tribes of horse-riding nomadic pastoralists. The dominant group of the tribes was the Xiongnu. Modu Chanyu of the Xiongnu formed an assembly of nomadic tribes and became the Chanyu (the supreme ruler, Son of the Heaven) and the Emperor of the Xiongnu Empire (209 B.C.–A.D. 93), located in modern-day Mongolia. The fixed dwelling of the Chanyu was Karakorum, which later became the capital city of Genghis Khan's Empire (Grousset, 2007). The Xiongnu Empire threatened the Chinese Han Dynasty (206 B.C.–A.D. 220) founded three years later. The Xiongnu Empire stretched beyond the borders of Mongolia. They were known to be active in the areas of Southern Siberia including Transbaikal region, Western Manchuria, and the Chinese provinces of Inner Mongolia including Ordos, plus Gansu, and Xinjiang. Their name, Xiongnu, may be related to the name known to the Greco-Roman world as Huns, but the identification of the two groups is not certain. Although the Xiongnu has been suggested to be Turco-Mongolian (Grousset, 2007), the language of the Xiongnu has been proposed as Yeniseian, Iranian, Turkic, or Mongolic (Adas, 2001; Di Cosmo, 2002).

The entire Xiongnu population seem not to link to one ethnicity, because the Empire ruled over a vast territory, including diverse nomadic tribes, for 300 years. A complex system of social stratification ranging from elites to an underclass of war captives was suggested to be main-

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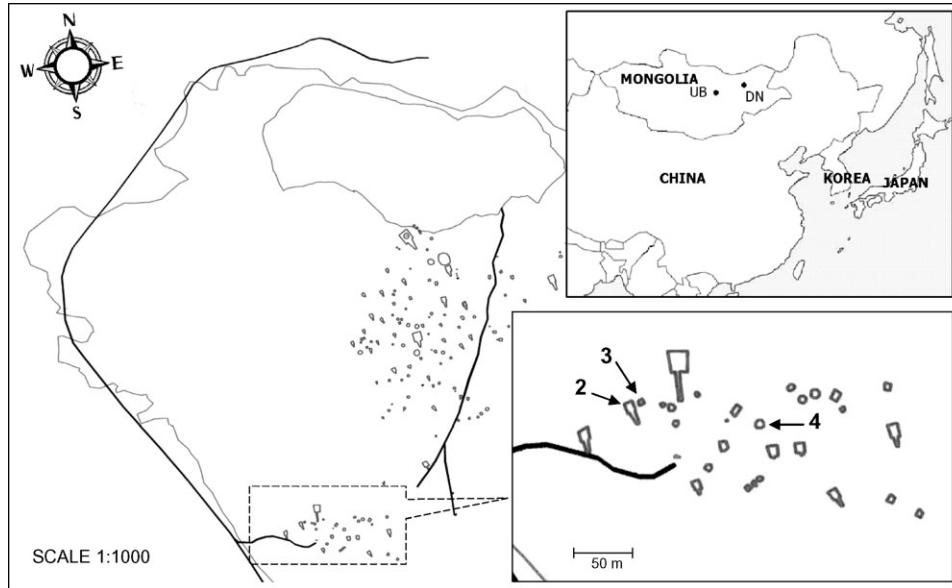


Fig. 1. Location of Duurlig Nars of Mongolia and map of the tombs. The numbers in the lower inset figure indicate the numbers of tombs from which the ancient skeletal remains of this study were excavated. UB, Ulaanbataar; DN, Duurlig Nars.

tained under the Xiongnu polity (Honeychurch et al., 2007; Popova, 2007). The Xiongnu were first described in Chinese texts as early as the 4th century B.C. (Minajev, 1996). Because they had no writing, an important evidence of their authority is the elite Xiongnu cemeteries. Most of the Xiongnu tombs have been found in Mongolia, indicating Mongolia as the heartland of Xiongnu Empire (Ochir, 2007). Duurlig Nars site is one of the six largest Xiongnu cemeteries found in Mongolia. Because of the many large tombs, the Xiongnu cemetery has been regarded as an elite cemetery, perhaps including even the remains of the Chanyu. But, there have been no attempt of excavation despite much attention to the cemetery.

The Kurgan expansion hypothesis explains the Indo-European expansion from the Volga steppe region (Gimbutas, 1973; Mallory, 1989). The paternal Y-chromosome single nucleotide polymorphisms (Y-SNP) R1a1 is considered as an Indo-European marker, supporting Kurgan expansion hypothesis (Zerjal et al., 1999; Kharkov et al., 2004; Haak et al., 2008). Recent finding of R1a1 in the Krasnoyarsk area east of Siberia marks the eastward expansion of the early Indo-Europeans (Keyser-Tracqui et al., 2009). R1a1 was not found in Scytho-Siberian skeletons from the Sebyste site of Altai Republic or in Xiongnu skeletons from Egyin Gol of Mongolia (Keyser-Tracqui et al., 2009).

Ancient DNA (aDNA) study methods are being applied to a wide variety of anthropological questions (Kaestle and Horsburgh, 2002). The analysis of aDNA is difficult due to the inability to amplify the material to a significant degree and due to the contamination with modern

DNA (Pääbo et al., 2004; Mulligan, 2005; Hunter, 2006). Several optimized aDNA study methods have now been introduced (Rohland and Hofreiter, 2007; Meyer et al., 2008). Recently, we have developed a further improved method (Kim et al., 2008).

To study the origin of the human remains in the elite Xiongnu cemetery, we have analyzed Y-chromosomal and mitochondrial haplogroups as well as autosomal short tandem repeats (STRs) from three human skeletons of the Xiongnu cemetery of 2,000 years ago at Duurlig Nars. We determined paternally transmitted Y-haplogroups to the subclade level. We also determined maternally inherited haplogroups by identifying the polymorphisms of HV1 (hypervariable region 1), HV2, and several coding regions of mitochondrial DNA (mtDNA). Kinship was analyzed with autosomal STRs. The genetic analysis of human remains from the cemetery could help gain some insight into the Xiongnu society.

MATERIALS AND METHODS

Site

In 1974, Mongolian archeologists discovered a Xiongnu cemetery at Duurlig Nars in Bayan Adarga sum north of Khenti Aimag, 500 km northeast of the capital of Mongolia, Ulaanbaatar. Khenti is the homeland of Genghis Khan. The Duurlig Nars cemetery was excavated in 2006–2007 by a Korean–Mongolian joint research team. More than 200 tombs were found in the Xiongnu cemetery at Duurlig Nars (see Fig. 1). The cemetery is located on a broad slope with high mountains to the east and steppes and knolls north and south. Long-lasting cold winter, cool summer, small precipitation, high-temperature fluctuation, and relatively high number of sunny days a year are characteristics of Mongolian climate. Khenti Aimag has a favorable environment for living, including three major rivers (Onon, Tol Gol, and Kherlen) and dense forest. The average temperature of the year is 0.9°C in Khenti Aimag. The annual average tem-

Abbreviations

DN	Duurlig Nars
HV1	hypervariable region 1
HV2	hypervariable region 2
mtDNA	mitochondrial DNA
NJ	Neighbor joining
UB	Ulaanbataar.

TABLE 1. Summary of archeological, anthropological, and molecular data

	Tomb number		
	2	3	4
Code	MNX2 male	MNX3 West Eurasian male	MNX4 female
Preservation	Poor	Excellent	Excellent
Sample	(1) Right upper second, third molar (2) Left upper first, second molar	(1) Right tibia (2) Right fibula	(1) Right femur (2) Right femur
Age at death	30–40	60–70	20–30
Burial position	Distorted	Distorted (North-West?)	Distorted (South?)
Archeological period	Iron	Iron	Iron
Archeological date	100BC–100AD	300BC–100BC	300BC–100AD
Sex			
Archeological	Male	Male	Female
Anthropological	Male	Male	Female
Molecular	Male	Male	Female

perature in January, the coldest month, is -30 – 34°C . The average temperature in July, the warmest month, is lower than 15°C . The different ancient nomadic groups left various historical remains behind in Khentii Aimag, such as petroglyphs from the paleolithic to the recent, deer stones, tombs from Xiongnu, Turk, and Mongolian periods, and ancient palaces.

Korean–Mongol archeologists excavated one \square -shaped (Tomb No. 2) and two rectangular tombs (Tomb No. 3 and 4). According to archeological evidence, Tomb No. 2 with a large dromos entryway was used between the 1st c. B.C. and the 1st c. A.D. and Tomb No. 3 between the 3rd c. B.C. and the 2nd c. B.C. Tombs were separated by piled-up stones. Most of them were disturbed by pillaging at some point in the past. Despite pillaging, many valuable funeral goods remained there. Further analysis is in progress. The main funeral goods are a chariot, a golden necklace, a silver spoon, a wooden parapet, a red-walled coffin decorated with thin golden plates, and bronze and iron artifacts. The golden necklace was only the second ones to be found in Xiongnu excavations in Mongolia. The first one was found in the Noyon Mountain (Tseveendorj, 1985). Tomb No. 2 has a dimension of 9×11 m with a large 15-m dromos entryway vestibule and is 8-m deep. About 22 animal bones, mainly skulls and limb bones of 12 horses and 10 goats and sheep, were found in an arranged pattern on the third terrace. The associated artifacts were a golden necklace, a black lacquered chariot, a horse-shaped gold ornament, strips of gold foil decorating wooden coffin, a silver spoon, several small objects of lapis lazuli, a Chinese bronze mirror, and many iron items with some bronze and gold parts. Tomb No. 3 was located 2-m east from Tomb No. 2. It is 6×6 -m square-shaped and 3.5-m deep and is not considered to be a satellite tomb of Tomb No. 2. Three ram skulls were found in Tomb No. 3. The burial goods were two round jars, a lamp, a Chinese bronze mirror, gold foil, gold belt ornaments, an antler, a bronze ring, and an iron arrowhead. Tomb No. 4 lies 150-m east from Tomb No. 2 with a 9×10 -m square-shape and is 5-m deep. The burial goods were gold foil decorating a wooden frame, a large vessel with horse bones wrapped by a silklike cloth, and a lamp-shaped pottery (Chang, 2007).

Ancient human samples

The associated culture, the time period, the estimated age, and the morphological sex of the specimens studied are presented in Table 1. Metric skull traits are presented (Fig. S1 and Table S1). All procedures were car-

ried out by Asians in a restricted clean room dedicated for ancient human DNA study and under sterile conditions, using UV-irradiated gowns with head and leg covers, latex gloves, and face- and mouth-masks. Autoclaved or presterilized materials were used. Work places, containers, and pipette surfaces were cleaned by bleach and UV irradiation at 254 nm. All steps were carried out in a fume hood and a laminar airflow clean bench. Extraction blanks and template blanks were included through the entire process. DNA extraction, polymerized chain reaction (PCR) preparation, and post-PCR works were performed in separate rooms. Sterile aerosol-barrier pipettes and pipette tips were used throughout all the manipulations. Different bones or different parts of the bone were analyzed for DNA information. We followed a published protocol (Kemp and Smith, 2005) to eliminate any potential DNA contamination on the bones. In brief, bone surfaces were removed. Bone fragments were immersed in bleach and irradiated with UV. Bone powders were made with Mixer Mill MM301 (Retsch). Ancient genomic DNA extraction was carefully carried out according to a published protocol (Kim et al., 2008). In brief, bone powders were incubated in an extraction buffer. After incubation of the supernatant with silica particles, the silica extracts were purified using ion-exchange columns with QBT, QF, and QC buffers (Qiagen). Elutes were concentrated with Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore) and Microcon YM-30 Centrifugal Filter Unit (Millipore).

mtDNA analysis

HV1, HV2, and several coding regions were analyzed. PCR was performed in a 20- μl reaction mixture containing 2- μl template DNA, 2- μl $10\times$ PCR buffer (ABI), 0.2 mM dNTP mix, 2 mM MgCl_2 , 1 μM each primer (Table 2), 1 mg/ml BSA (NEB), and 0.8 U AmpliTaq Gold polymerase (ABI). A GeneAmp[®] PCR system 9700 (ABI) was used with the following conditions: 95°C for 10 min, 40 cycles of 30 s at 95°C , 1 min at the annealing temperature, 1 min at 72°C , followed by a final extension step of 7 min at 72°C . Low-quality amplicons were reamplified in a nested PCR reaction under the same PCR cycling condition as above but with the first PCR product diluted 1:50 and 25 cycles. PCR products were purified using Qiaquick PCR purification kit (Qiagen) and bidirectionally sequenced. Sequencing results were analyzed using SeqMan[™] II software (DNASTAR). Sequences were compared to revised Cambridge reference sequence (rCRS) (Andrews et al., 1999). Haplogroups of aDNA

TABLE 2. Primers for the amplification of mtDNA HV1, HV2, and some coding regions

Regions ^a	Primer name ^b	Sequence (5' → 3')	AT (°C) ^c	Size (bp)	Reference
15,977–16,399	F15971	TAACTCCACCATTAGCACC	60	440	Edson et al., 2004
	R16410	GAGGATGGTGGTCAAGGGAC			
	FN15977	CCACCATTAGCACCCAAAGC	60	423	Edson et al., 2004
	RN16399	TCAAGGGACCCCTATCTGA			
	FA1	CCCCCCCCCAT			
	FA2	CCCCCCCCGTTA			
29–381	RA	GGGGGGGGGTT	56	378	Edson et al., 2004
	F15	CACCCTATTAACCACTCACG			
	R389	CTGGTTAGGCTGGTGTAGG	56	353	Edson et al., 2004
	FN29	CTCACGGGAGCTCTCCAT			
5,055–5,324 (D)	RN381	GCTGGTGTAGGGTTCTTTG	57	286	Present study
	F5047	TTCTACCGTACAACCCTAACATAAC			
	R5313	AGGAGGGTGATGGTGGCTAT	57	270	Present study
	FN5055	TACAACCCTAACATAACCATTCTTA			
2,934–3,239 (D4)	RN5324	GATGGTGGCTATGATGGTG	58	342	Present study
	F2907	TTGACCAACGGAACAAGTTACC			
	R3229	CGGGCTCTGCCATCTTAACA	56	306	Present study
	FN2934	GATAACAGCGCAATCCTATCT			
12,243–12,458 (U)	RN3239	CCATCTTAACAAACCCTGTTCT	60	264	Present study
	F12203	TACCGAGAAAGCTCACAAGAAGT			
	R12447	AGGTGGATGCGACAATGGAT	60	216	Present study
	FN12243	TGTCTAACAAACATGGCTTCTCA			
15,806–15,990 (U2e)	RN12458	GCGACAATGGATTTTACATAATGG	60	290	Present study
	F15754	CTGAATCGGAGGACAACCAGTA			
	R16023	TCTGCTTCCCCATGAAAGAAC	60	185	Present study
	FN15806	GCATCCGTACTATACTTCACAACAA			
	RN15990	GGTGCTAATGGTGGAGTTAAAGAC			Present study

mtDNA haplogroup nomenclature is based on the updated mtDNA haplogroup tree (van Oven and Kayser, 2009).

^a Numbers indicate nucleotide positions based on the revised Cambridge reference sequence (Andrews et al., 1999), the haplogroup to determine is shown in the parenthesis.

^b F, forward primer; R, reverse primer; N, primers for nested PCR and sequencing; A, sequencing anchor primer in case of failed sequencing reaction due to the presence of a polycytosine tract.

^c Annealing temperature.

were initially assigned with HV1 and HV2 polymorphisms using the well-established web-based programs, Haplogroup Prediction Tool and mtDNAMANAGER. We further analyzed diagnostic coding region SNPs for corresponding haplogroups based on a recently updated global human mtDNA tree (van Oven and Kayser, 2009). Coding region SNPs with no homoplasy (Behar et al., 2007) were used. Possible abnormal mutations were checked with GenBank database and Genographic project database.

The most closely related haplotypes of the three aDNAs among 8,424 mtDNA sequences from the GenBank database were investigated by constructing a Neighbor-joining (NJ) tree (Saitou and Nei, 1987) with concatenated HV2 (nucleotide position, NP 47-360) and HV1 (NP 16,024-16,380) sequences by using Clustal W 2.0.11 (Larkin et al., 2007). The NJ tree was drawn using MEGA version 4.1 (Kumar et al., 2008) (Fig. S2). mtDNA sequences proximate to the aDNA sequences were further analyzed by reconstructing a bootstrapped NJ tree calculated from 1,000 resamplings of the alignment data (see Fig. 2). Proximate mtDNA sequences were compared to rCRS. Sequence similarities to aDNA sequences were determined (Tables S2–S5).

Real-time PCR for quantification of ancient mtDNA molecules

Real-time PCR assay was carried out to quantify the number of mtDNA molecules in the aDNA extracts of MNX3 European male and MNX4 female samples as follows. Two different size fragments (440 and 221 bp) within the HV1 mtDNA region were assayed using

primer sequences: F15971/R16410 for the 440-bp fragment (Table 2); and F16190 (5'-CCC CAT GCT TAC AAG CAA GT-3')/R16410 for the 221-bp fragment. Real-time PCR amplification was performed in 20 μ l with 2 μ l of extracts, 0.5 μ M each primer, 0.8 mg/ml BSA, and LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) according to the manufacturer's instructions. Thermal-cycling conditions were preincubated for 15 min at 95°C, 42 cycles of 95°C for 10 s, 60°C for 3 s, and 72°C for 30 s, and a melting curve cycle (10 s at 95°C, 60 s at 65°C, and temperature increase from 65 to 95°C with a temperature transition rate of 0.1°C/s). SYBR-green uptake in double-stranded DNA was measured on a LightCycler 2.0 system (Roche). Product specificity was controlled using the melting-curve analysis of the Roche LightCycler software 4.05 (Roche). Ten-fold serial dilutions (from 5×10^5 to 50 copies) of purified 440-bp PCR product quantified by NanoPhotometer (IMPLEN) were included in each experiment to generate standard curve. At least two "no-template-control" were included with each experiment. The real-time PCR experiment was performed in at least duplicate. Analysis of the data was performed using LightCycler software 4.05 to generate individual standard curves from each experiment and to calculate the DNA amount from each unknown sample. Standard curves showing the correlation coefficient of the trendline higher than 0.95 were used.

Y-SNP analysis and autosomal STR analysis

A set of 16 biallelic markers for Mongolia were studied based on the global Y haplogroup distribution and

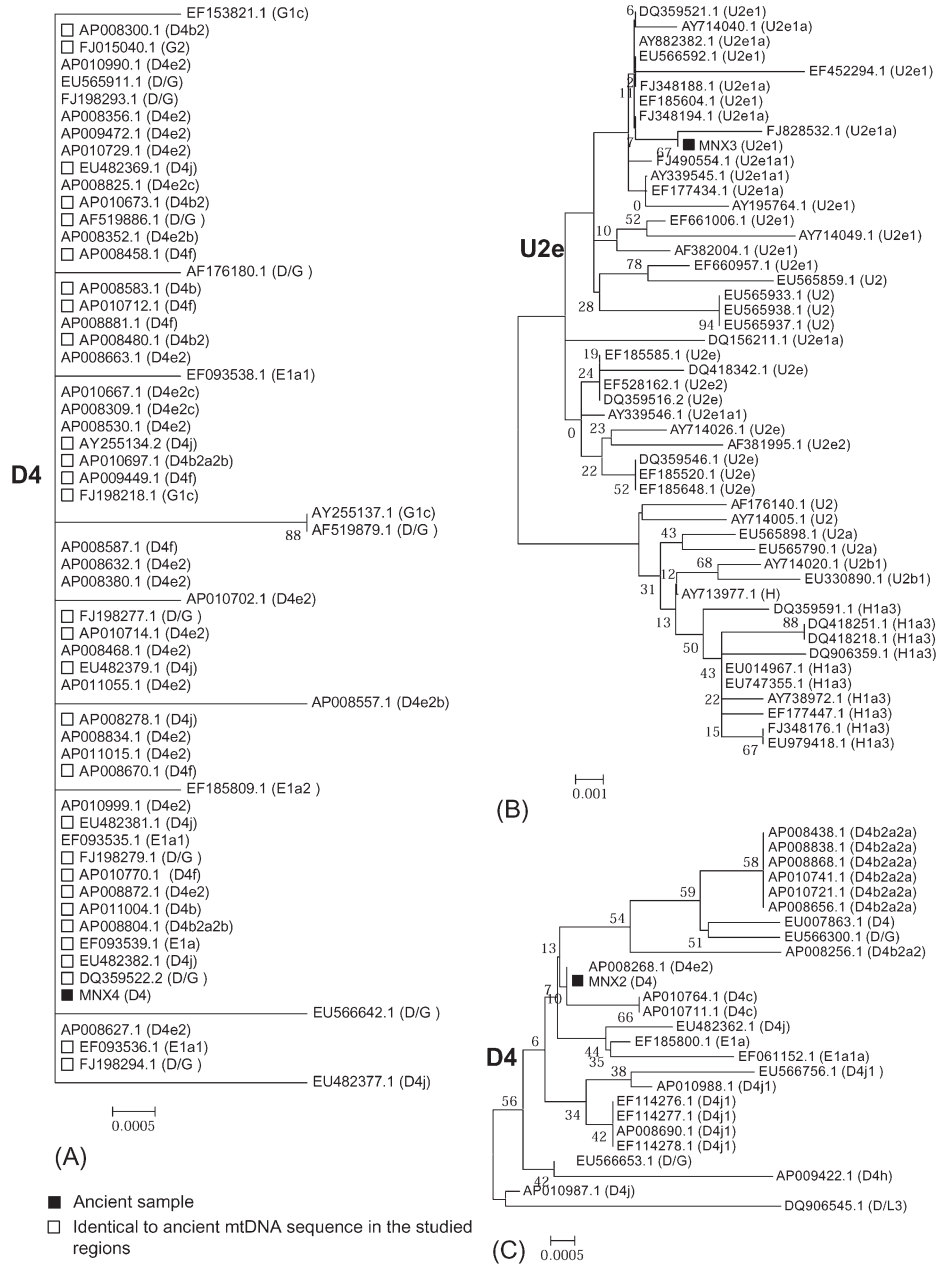


Fig. 2. Neighbor-joining (NJ) trees of proximate mtDNA sequences to three ancient Mongolian samples based on the concatenated partial HV1 and HV2 control region mtDNA sequences. Taxons are labeled as the GenBank accession number of their mtDNA sequence and their haplogroup in parenthesis. The numbers at the nodes represent bootstrap percentage value estimated from 1,000 replications. A predominant haplogroup is shown in big bold over the branch.

recently revised markers (Table 3) (Jobling and Tyler-Smith, 2003; Karafet et al., 2008). All the known primers except for one were redesigned for amplicons between 100 and 230 bp. The target markers were amplified in monoplex or multiplex reactions. The markers in multiplex reactions were RPS4Y₇₁₁, M174 and M231 for multiplex I, M207, M304, and M242 for multiplex II, and M38, M217, and M210 for multiplex III. PCR was performed in a 10- μ l final volume composed of 1 \times PCR Gold Buffer (ABI), 2 mM MgCl₂, 0.2 mM dNTP, 1 mg/ml BSA (NEB), 0.4 U AmpliTaq Gold DNA polymerase (ABI), 0.75 μ M primer, and 4 μ l aDNA template. Thermal cycling consisted of a first denaturation step at 95°C

for 11 min followed by 45 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 7 min. Each marker was amplified again in a nested PCR reaction under the same cycling condition as above but with a 1:50 dilution of the first PCR product and 25 cycles. Amplicons were sequenced in forward or reverse direction. Y haplogroup was determined with recently revised Y haplogroup tree (Karafet et al., 2008).

For the autosomal STR analysis, triplicate amplifications were performed with AmpFlSTR[®] Minifiler[™] PCR Amplification Kit (ABI). PCR was performed according to manufacturer's protocol but with 32 amplification cycles. PCR products were analyzed with the ABI Prism

TABLE 3. Primers for the amplification of Y-SNP markers used in this study

Marker (haplogroup ^a)	Sequence (5' → 3')		Size (bp)	Mutation	
	Forward	Reverse		Site	Change
M175 (O)	TAGTACCCAAATCAACTCAACTCC	CAGCATTTCAGTTAGCCTTGA	219		
Nested	GCACATGCCTTCTCACTTCT	TTCAGTTAGCCTTGATTGACTGT	164	22	5 bp Del
RPS4Y ₇₁₁ (C)	CAGGGCAATAAACCTTGGAT	CCACAAGGGGGAAAAAACAC	188		
Nested	GGGCAATAAACCTTGGATTT	CATTAAGAAACGAGAATTCCTG	167	22	C → T
M174 (D)	CACTGTGCGTTCTTCTCCGTCAC	TGACTAGAAGGTCTCGGAGATGC	217		
Nested	CTCCGTCACAGCAAAAATGTAC	CCCATCTTGCAAGGAAAAAGT	162	93	T → C
M231 (N)	GGAAAATGTGGGCTCGTTT	GAATGGTGGCCAGAGTCTTTTAC	207		
Nested	GGCTCGTTTTAATTATATTCATAT	GGCCAGAGTCTTTCACATCAT	190	89	G → A
M207 (R)	ACTATGGGGCAAATGTAAGT	GCTGTTCGCTGCTACGAAT	162		
Nested	GGCAAATGTAAGTCAAGCAAGAA	CGCTGCTACGAATCTTTAATCTTA	149	29	A → G
M304 (J)	GGTATTGGGGTAGGCAAAGA	CCTTCAGGCTTCTAGCTTCATC	207		
Nested	TGGGGTAGGCAAAGAAAAAG	GGCTTCTAGCTTCATCTGCATTGT	196	88	A → C
M242 (Q)	TCTACGGCATAGAAAGTTTGTG	GCTTTAAGGGCTTTCAGCAT	168		
Nested	TGTGCAAAAAGGTGACCAA	GGCTTTCAGCATAATACCTTAC	142	26	C → T
M173 (R1)	CTTAAATTCGAAGGGCATTTAG	AAAAGTCAACAGGGCTTTG	230		
Nested	CAATTCGAAGGGCATTTAGAA	CTTTACCTTCACGCCTTCA	165	22	A → C
M124 (R2)	CAAATTCCTGGGCAACAC	ATCAACTTCTTCCCTCAACAT	183		
Nested	GCAACACCAGAATCTAACAAAGC	TCTTCCCTCAACATAGTTATCTC	165	51	C → T
M8 (C1)	GTGCTCAGTATCACAAACAC	CGAGGCTGACAGACAAGTC	200		
Nested	TCAATAACTTGGACTGGG	GCTGACAGACAAGTCCACCA	151	23	G → T
M38 (C2)	CAGGAGATCTGTTGGCTACTG	GGAGCTGGCACATCTGTCA	182		
Nested	GGGTATGGCAATGGTATGTA	AGCTGGCACATCTGCATAAT	151	63	T → G
M217 (C3)	CAAAGAAAGGCCAGTATCTC	GCTGCTGTGGCTTTCATCAAAA	185		
Nested	GCCAGTATCTCCAAAATCCT	TTTTTATGTATTTTCTCTTGAA	151	122	A → C
M210 (C4a)	CAGAAGCCGAGTAGGAAGCTAT	TGTATTTATCTTCCCTGCATTC	124		
Nested	CCGAGTAGGAAGCTATCTATGACT	TATCTTCCCTGCATTCAGAAGT	112	38	A → T
SRY _{10831.2} (R1a)	TCATTTGCCTTTCTCGGATA	CAACAGGAAATCCCTTACAAGA	189		
Nested	ACATGGGATCATTCAGTATCTG	AAATCCCTTACAAGATGAGAAA	154	52	G → A
M343 (R1b)	AGGAGGCGGTGTCTGATT	ACCCACATATCTCCAGG	153		
Nested	AAGGCTCAGGGTATTGGTT	CCCACATATCTCCAGGTG	127	108	C → A
M17 (R1a1)	GGTCAAAAAGAGGCGTAGATAC	CAAAAATAGTTTGGCCACTTAAC	174	126	G Del

^a Y-SNP haplogroup nomenclature used here is based on the revised haplogroup tree (Karafet et al., 2008).

310 automatic sequencer (ABI). More than six repeats were done to identify reproducibility (Schmerer et al., 1999). Genetic relationships and ethnic affiliations were investigated with the DNA-VIEW software.

Authenticity assessment

The classical-stringent standards for the authentication of aDNA were followed. mtDNA quantification of aDNA extracts determined by duplicate real-time PCR revealed that two MNX3 extracts carried on average 2,550–5,500 and 92,500–122,000 copies per gram of bone powders for 440 and 221-bp mtDNA HV1 fragment, respectively (Table S6 and Fig. S3); two MNX4 extracts had on average 1,250–1,522 and 34,750–35,750 copies per gram of bone powders for 440 and 221-bp mtDNA HV1 fragment, respectively. A horse mtDNA fragment was amplified from an associated horse remain only with the primers for horse mtDNA amplification but was not with the primers for human mtDNA (Fig. S4). These results strongly demonstrate that the potential human DNA contamination has been successfully resolved in this study, and also the genuine target DNA was successfully obtained. The cloning of amplified ancient mtDNA HV1 fragments from each of the MNX3 West Eurasian male and MNX4 female samples showed no significant sequence variation among the 10 independent clones of each sample except poly-C heteroplasmy compared to amplicon-derived sequences from the MNX3 West Eurasian male sample (Table S7); this kind of heteroplasmy does not affect the haplogroup determination in this

study and has been reported to be observed normally and frequently (Santos et al., 2008). None of the samples shared the same mtDNA or STR genotype with any other sample or with laboratory workers and archeologists (Table S8). We tested two or more separate extracts from one or two different bones. Extraction and template blanks were included in every PCR procedure and no positive was detectable. aDNA samples were reproducible in all the analyses. Results were consistent between haplogroup and subhaplogroup markers in mtDNA and Y-SNP analyses. There were no unreported, ambiguous, or heteroplasmic variations in any of the sample data. Independent replication for the mtDNA, Y-SNP and autosomal STR analysis of the ancient samples, MNX3 Eurasian male, and MNX4 female was all successfully done in a second laboratory (Lee's Laboratory, Department of Life Science, College of Natural Science, Chung-Ang University). These findings are in favor of the authenticity of the amplified products. The second-laboratory replication of MNX2 male sample demonstrated only a small fragment of mtDNA HV1 (221 bp). PCR amplifications for the large mtDNA fragments (440 and 378 bp), Y-SNP, and autosomal STR analysis were failed. The partial HV1 sequencing data obtained from the second laboratory were consistent with those from the two independent extracts from the first laboratory. It is unlikely that there were potential contamination problems in the second laboratory. We presume that the replication could not be completed due to the lack of available aDNA extracts. The genetic data of MNX2 male should be interpreted carefully with respect to authenticity.

TABLE 4. Y-SNP haplogroup and mtDNA sequence variations in three ancient DNA samples

Samples	Y-SNP		mtDNA				
	Haplogroup	Haplogroup	HV1 (NP 15997–16380)		HV2 (NP 47–361)		Coding region
Determined by the first laboratory							
MNX3	R1a1	U2e1	16051 16093 16129C 16182C 16183C 16189 16362	73	152 217 263 315+C 340	12308, 12372, 15907	
MNX4	NA ^a	D4	16223 16362	73	263 309+C 315+C	3010, 5178A	
MNX2	C3	D4	16223 16311 16362	73	263 309+C 315+C	3010, 5178A	
Determined by the second laboratory							
MNX3	R1a1	U2e1	16051 16093 16129C 16182C 16183C 16189 16362	73	152 217 263 315+C 340	12308, 12372, 15907	
MNX4	NA	D4	16223 16362	73	263 309+C 315+C	3010, 5178A	
MNX2	ND ^b	ND	(16223 16311 16362) ^c		ND	ND	

Positions are numbered according to the revised Cambridge reference sequence (rCRS) (Andrews et al., 1999). These are transitions from the reference sequences unless a base is explicitly indicated. Suffixes indicate transversions and “+” indicates insertions recorded at the last possible site. For each haplogroup, characteristic mutations are shown in bold.

See Table 3 for the Y-SNP haplogroup determination.

^a Not available, female.

^b Not determined because of amplification failure.

^c Determined by a small fragment (NP 16190–16410) amplification.

RESULTS

MtDNA analysis

MtDNA sequences of HV1 (NP 15,991–16,390 or NP 15,997–16,380), HV2 (NP 35–369 or NP 47–361), and coding regions, NP 12,266–12,434, NP 15,831–15,966, NP 2,956–3,217, and NP 5,080–5,305, were reproducibly obtained and are available at GenBank (accession numbers: GQ145583–GQ145594). Compared to rCRS, MNX3 West Eurasian male had six (HV1) and five (HV2) variable NPs; MNX4 female had two (HV1) and four (HV2) variable NPs; MNX2 male had three (HV1) and four (HV2) variable NPs (Table 4). The HV1 sequences of the three ancient samples were different from one another. The HV2 sequences of MNX 2 male and MNX4 female were identical. The HV1-based Haplogroup Prediction Tool determined MNX3 male as haplogroup U, and MNX 2 male and MNX4 female as D. mtDNAMANAGER determined MNX3 West Eurasian male as haplogroup U2e, MNX2 male and MNX4 female as D4/G. Coding gene motifs confirmed the predicted haplogroups. MNX3 West Eurasian male carried haplogroup U coding gene mutations A → G transition at NP 12,308 and G → A transition at NP 12,372, and U2e coding gene mutation A → G transition at NP 15,907. MNX3 carried U2e1 defining mutations T → C transition at NP 217 on HV2 (Table 4) (Palanichamy et al., 2004; Achilli et al., 2005; van Oven and Kayser, 2009). MNX3 West Eurasian male determined with a NJ phylogenetic tree was a close neighbor to U2e1 (Figs. 2 and S2). The most similar sequences to MNX3 West Eurasian male were a Hutterite origin DNA (GenBank No. FJ348188.1) with 1-bp differences from each of HV1 and HV2 of MNX3 and a Finnish origin DNA (AY339545.1) with 2 bp and no difference at HV1 and HV2, respectively (Tables S2 and S5). The origins of sequences that showed 3-bp differences in HV1 and HV2 from MNX3 West Eurasian male sequence were Spain, Hungary, Italy, and two unidentified ones. One sequence (Serial No. 3,890) of unknown origin among the 50,033 HV1 sequences in the Genographic project database was identical to the MNX3 West Eurasian male HV1 sequence. A search of 19,626 haplotypes from a global mtDNA control region database from the Armed Forces DNA Identification Laboratory found no exact matches. Two samples differed at one and two polymorphisms: a

sample from UAE lacked the 16093C variant, and a sample from Afghanistan matched the MNX3 West Eurasian male and contained the 309.1C and 309.2C length polymorphisms from HV2 (Table S5).

MNX2 male and MNX4 female carried haplogroup D coding gene mutation C → A transversion at NP 5178 and D4 coding gene mutation G → A transition at NP 3010 (Table 4) (Kong et al., 2006; van Oven and Kayser, 2009). NJ tree analysis showed that these two ancient samples were most closely related to D4 people primarily composed of East Asians (Figs. 2 and S2 and Tables S3 and S4). Twenty-nine haplotypes were identical to MNX4 female, but none was identical to MNX2 male (Figs. 2 and S2 and Table S5).

Haplogroup Prediction Tool and mtDNAMANAGER correctly and consistently determined haplogroup U samples with very few incorrect assignments (Table S2). However, Haplogroup Prediction Tool resulted in incorrect determinations for haplogroup D (Tables S3 and S4). These results show that haplogroup determination with HV1 sequence variation (Haplogroup Prediction Tool) has a limitation and mtDNAMANAGER is preferable for Asian mtDNA and indicate that haplogroup determination, based only on control region variations, has a potential risk of haplogroup misdiagnosis; the examination of diagnostic coding region mutations should be considered for the reliable and precise haplogroup determination.

Y-SNP analysis

We analyzed a set of 16 Y-SNP biallelic markers for the two ancient male-determined samples. MNX3 West Eurasian male was initially determined as haplogroup R (M207). Subsequent analyses revealed MNX3 West Eurasian male as R1 (M173), R1a (SR_{Y10831.2}), and R1a1 (M17) (Fig. S5). The MNX2 male haplogroup was determined as C (RPS4Y₇₁₁) and C3 (M217) (Fig. S6).

Autosomal STR analysis

Autosomal STR typing showed complete allelic profiles (Table 5). Kinship analysis with DNA-VIEW excluded a close parentage relationship among three ancient subjects. Comparing MNX2 male with MNX3 West Eurasian

TABLE 5. Summary of allelic profiles obtained with Minifiler™ PCR Kit from three ancient DNA samples

Samples	Extractions	Amelo	D13S317	D7S820	D2S1338	D21S11	D16S539	D18S51	CSF1PO	FGA
MNX3 West Eurasian male	Rt tibia 1	X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
	Rt tibia 2	X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
	Rt Fibula	X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
	Rt Tibia 3 (second Lab)	X/Y	10/12	10/12	23/24	28/29/31.2	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
		X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11	23/25
Consensus profile MNX4 female	Rt Femur 1	X/Y	10/12	10/12	23/24	28/29	10/11	14/15	11/11	23/25
		X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
		X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
	Rt Femur 2	X/X	11/12	12	20/24	30/31.2/32.2	9/13	17/21	10/12	22/23
		X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
		X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
	Rt Femur 3	X/X	11/12	12	20/24	31/31.2/32.2	9/13	17/21	10/12	22/23
		X/X	11/12	12	20/24	31/32.2	9/13	17	10/12	22/23
		X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
	Rt Femur 4 (second Lab)	X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
		X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
		X/X	11/12	12	20/24	31/32.2	9/13	17/21	10/12	22/23
Consensus profile MNX2 male	Rt. upper	X/X	11/12	12/12	20/24	31/32.2	9/13	17/21	10/12	22/23
		X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
		X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
	Second, third	X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
		X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
		X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
	Lt. upper	X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
		X/Y	9/11	8	20/25	28.2/29/38.2	11/14	13/18	11	22
		X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
	First, second	X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
		X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
		X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22
Molar	X/Y	9/11	8/8	20/25	28.2/38.2	11/14	13/18	11/11	22/22	
	X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22	
	X/Y	9/11	8	20/25	28.2/38.2	11/14	13/18	11	22	

Consensus profiles are shown in bold.

male and MNX4 female shows the affirmative evidence that they are not closely related. A likelihood ratio (LR > 10) supports unrelated versus half-sibling for MNX2 male versus either of the others. In this respect, half-sib is a reasonably representative weak relationship; it is the same calculation as for uncle/nephew or grandparent/grandchild. Furthermore, the evidence supporting unrelated versus closely related (sibling or parent/child) is very strong (LR > 500). As between MNX3 West Eurasian male and MNX4 female, autosomal DNA gives strong evidence that they are not immediate relatives, but only weak evidence against a half-sibling (uncle etc.) relationship and no evidence either way as regards being cousins.

Autosomal DNA can also be used as evidence of population origin using the principle that the likelihood is greater for a population in which the alleles of a profile are more common (Brenner, 2006). Calculation by DNAVIEW shows that the autosomal profile of MNX3 West Eurasian male is 14 times more probable from a Brahmin Indian than from a modern Caucasian (Table 6).

DISCUSSION

Xiongnu cemeteries have been found at Noin-Ula, Bor Bulagin Am, Golmod, Golmod-II, Takhilt Khotogo, Egyin Gol, and Duurlig Nars in Mongolia. Duurlig Nars has been considered as an elite cemetery among those sites in Mongolia. The excavation of three tombs in Duurlig Nars site has proved this through the recovery of various luxury items such as gold ornaments. This archeological

TABLE 6. Relative support for each racial origin calculated by DNAVIEW software with the autosomal STR data of Mongolian ancient samples

	Ancient samples ^a		
	MNX4 male	MNX2 female	MNX3 West Eurasian male
Race	(mt: D4, Y: C3)	(mt: D4)	(mt: U2e1, Y: R1a1)
Brahmin Indian	1	16	14
Egyin Gol (Mongolia)	190	160	5.4
Korean	130	1	1.9
Caucasian	61	30	1

^a mt, mtDNA haplogroup; Y, Y-SNP haplogroup.

evidence demonstrates the social stratification of the Xiongnu Empire. In this study, genetic analysis was completed on three subjects from Duurlig Nars cemetery dating to 2,000 years ago. To the best of our knowledge, there has been no detailed and integrated molecular analysis of ancient human subjects using mtDNA haplogrouping, Y-SNP haplogrouping, and autosomal STR genotyping in the Xiongnu population. We report here for the first time the occurrence of the R1a1 haplogroup in the Xiongnu population in Northeast Mongolia.

The MNX3 West Eurasian male has R1a1 of Y-SNP and U2e1 mtDNA. R1a is the most common haplogroup in Europe (Malyarchuk et al., 2004; Kayser et al., 2005; Wetton et al., 2005; Fechner et al., 2008; Volgyi et al., 2009). It shows decreasing frequencies from North to South Europe (Wells, 2007) and from Central toward

TABLE 7. Frequencies of Y-SNP and mtDNA haplogroups in present populations

Region (locale, ethnic)	n	R (% , Y-SNP)			U (% , mtDNA)		Reference
		R1	R1a	R1a1	U2	U2e	
Poland	913	68.6	68.6				Kayser et al., 2005
Germany	1215	56.9	56.9				Kayser et al., 2005
UK (Caucasian)	142	55.6	6.3				Wetton et al., 2005
Russia (Europe part)	338	52.3	47.5		0.4		Fechner et al., 2008
	325				1.5		Malyarchuk et al., 2004
Hungary	215	45	25.6				Volgyi et al., 2009
Nepal	188		24.5				Gayden et al., 2007
India (Brahmins)	621			72.2			Sharma et al., 2009
Upper Caste	145			38.6			Sengupta et al., 2006
Upper Caste	225				>70	>15	Basu et al., 2003
East	131				6.1		Sahoo and Kashyap, 2006
Northwest	34				17.6		Quintana-Murci et al., 2004
Pakistan (Kalash)	176		24.4			15.9	Quintana-Murci et al., 2004
	44		3				Quintana-Murci et al., 2004
Mongolia (Khoton)	40			82.5			Katoh et al., 2005
Uzbekistan	20					5	Comas et al., 2004
Azerbaijan (East, West)	40					2.5	Quintana-Murci et al., 2004
Kyrgyz	20					5	Comas et al., 2004
Turkmenistan (Kurdish)	32					6.3	Quintana-Murci et al., 2004
Tajikistan (High Pamirs)	44					6.8	Quintana-Murci et al., 2004
Iran (Center, South)	42				4.8	2.4	Quintana-Murci et al., 2004

South Asia (Wells et al., 2001). R1a1 in Nepal (Gayden et al., 2007) and India (Sahoo and Kashyap, 2006; Thanseem et al., 2006; Sharma et al., 2009) has been suggested to be associated with migration of Indo-European people from Central Asia (Cordaux et al., 2004). The mtDNA haplogroup U2e has been found in most Central Asian populations (Comas et al., 2004). This haplogroup shows high frequencies in Turkmenistan, Tajikistan, and Kalash in Pakistan (Table 7) (Quintana-Murci et al., 2004). U2e is present in West Eurasia at 1% on average (Richard et al., 2007). The ancestral mtDNA haplogroup U2 is subdivided into U2e and U2i; U2e is known as a characteristic European haplogroup, and U2i as an indigenous haplogroup in India (Kivisild et al., 1999; Bermisheva et al., 2002; Basu et al., 2003; Quintana-Murci et al., 2004; Maji et al., 2008; Malyarchuk et al., 2008). U2e is also found in India but exclusively in caste populations, especially in the upper caste with a high frequency (Table 7) (Basu et al., 2003; Maji et al., 2008).

Kurgan people were nomadic peoples of the Volga steppe region, infiltrating Europe between the middle of the fifth and the second millennium BC. Kurgan people expansion would have resulted in the spread of the Indo-European language (Gimbutas, 1970). The Kurgan culture is divided into different subcultures and thought to succeed to the following cultures (Gimbutas, 1970; Hemphill and Mallory, 2004). Historical records and archaeology attest that Kurgan nomadic groups moved across Eurasia from North of the Black sea through Central and Inner Asia, to northeast Asia in a matter of centuries (Mair, 2005). Carriers of the Kurgan culture, believed to be Indo-European speakers, were also carriers of the R1a1 haplogroup (Keyser-Tracqui et al., 2009). R1a1 has thus been considered a marker of Indo-European contribution (Zerjal et al., 1999; Kharkov et al., 2004). R1a was found in Eulau, Germany of the Corded Ware Culture (Haak et al., 2008). R1a1 was predominant in the Krasnoyarsk area in southern central Siberia with the Andronovo, the Karasuk, the Tagar, and the Tachtyk cultures (Keyser-Tracqui et al., 2009).

Our research shows that the MNX3 ancient human sample was not that of an East Asian. There are previ-

ous reports of ancient mtDNA haplogroup U; U5 in China 1,400 years ago and U2 in Mongolia 2,000 years ago (Keyser-Tracqui et al., 2003; Xie et al., 2007). mtDNA haplogrouping of Egyin Gol only with HV1 was an incomplete analysis. Although nearly 11% of ancient skeletons in Egyin Gol Xiongnu graves have shown European haplogroups, the later study revealed that there was no R1a1 (Keyser-Tracqui et al., 2009). Although the European-like skeletons have been found in Xiongnu cemeteries, there is no direct genetic evidence that they were R1a1 Indo-European. R1a has a very high frequency in modern Brahmins, irrespective of linguistic and geographic affiliations, suggesting that it was the founder haplogroup for the population (Sharma et al., 2009). Recent studies show that Y-chromosome haplogroup R1a lineages may have their origins in North India (Sahoo et al., 2006; Sengupta et al., 2006). The haplogroup R1a1, in the recent study, might have its origin in an Indian upper caste system, Brahmins (Sharma et al., 2009). On the other hand, the high frequency of R1a lineages and haplogroup R1a1 among upper caste Brahmins may reflect an intrusion from the northwest with speakers of Indo-European languages. MNX3 West Eurasian male likely represents a Bronze Age migration from the Black Sea region and has a common Y-chromosomal heritage with the Brahmins of India. In our analysis of aDNAs, from other ancient human skeletons excavated in Mongolia, haplogroup R of Y-SNP occurred at 14% among 98 specimens (our unpublished data). The Xiongnu was once dominant in West China including the Silk Road and close to the territory of Scythians, known as the Indo-European, and also dominated the southern portion of Central Siberia. It is also possible that the MNX3 West Eurasian male might be the Scythian from West Siberia or Kurgan people from Siberia northwest to the Lake Baikal.

MNX2 male and MNX4 female belonged to D4 mtDNA. D is common in Northeast and Central Asia (Yao et al., 2002; Comas et al., 2004; Gokcumen et al., 2008). D4 is more common in Turkmenistan (Quintana-Murci et al., 2004) and Northeast Asia (Yao et al., 2002; Maruyama et al., 2003; Cheng et al., 2008; Jin et al., 2009). MNX2 male showed C3 of Y-SNP. C is one of major groups in

Asia. The C frequency is higher in Northeast Asia (Jin et al., 2003; Yoshida and Kubo, 2008). Buryat shows the highest frequency of C3 (Derenko et al., 2007). The C3 frequency is also high in Mongolia and Evenks (Derenko et al., 2007).

Different mtDNA and Y-SNP haplogroup and sequences excluded immediate kinship among the three aDNA samples. Although the both belong to D4, MNX4 female is different at one site (NP 16,311) from MNX2 male. NP 16,311 is considered as a mutational hotspot (Malyarchuk and Rogozin, 2004). Autosomal STR analysis with DNA-VIEW supports unrelatedness among the three subjects. The kit for degraded DNA has five loci in common with the AmpFI/STR profiler Plus Kit: D13S317, D7S820, D21S11, D18S51, FGA, and amelogenin. No matched case was found between our data and data from ancient Kurgan sites, Egyin Gol, and modern Mongolians (Keyser-Tracqui et al., 2006, 2009).

In conclusion, paternal, maternal, and biparental genetic analyses were done on three Xiongnu tombs of Northeast Mongolia 2,000 years ago. We showed for the first time that an Indo-European with paternal R1a1 and maternal U2e1 was present in the Xiongnu Empire of ancient Mongolia. Furthermore, the autosomal STR analysis of the sample also supported the non-East Asian origin of this individual, and he was closer to the modern Indian high caste than to other modern populations, but not greatly so. Another possibility for his origin is the Transbaikalian region and the Scythian territory. Artifacts in the tombs suggested that Xiongnu had a system of social stratification. The West Eurasian male in the Xiongnu Empire might show the racial tolerance of the Xiongnu. Despite signs of pillaging, many valuable funeral goods remained intact in the Xiongnu tombs. Distorting human remains sometimes after the funeral might be a funeral custom, although adequate archeological and historical evidence for this remains to be brought forward.

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APPENDIX

URLs

GenBank database: <http://www.ncbi.nlm.nih.gov/Genbank/>

Genographic project database: <http://www.national-geographic.com/genographic>

Haplogroup Prediction Tool: <http://nnhgtool.national-geographic.com/classify>

MtDNAManager: <http://mtmanager.yonsei.ac.kr>

DNA-VIEW software: <http://www.dna-view.com/dnview.htm>

Clustal W 2.0.11: <ftp://ftp.ebi.ac.uk/pub/software/clustalw/2.0.11/>

MEGA software version 4.1: <http://www.megasoftware.net/mega41.html>

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