



## Research

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# Identification of kinship and occupant status in Mongolian noble burials of the Yuan Dynasty through a multidisciplinary approach

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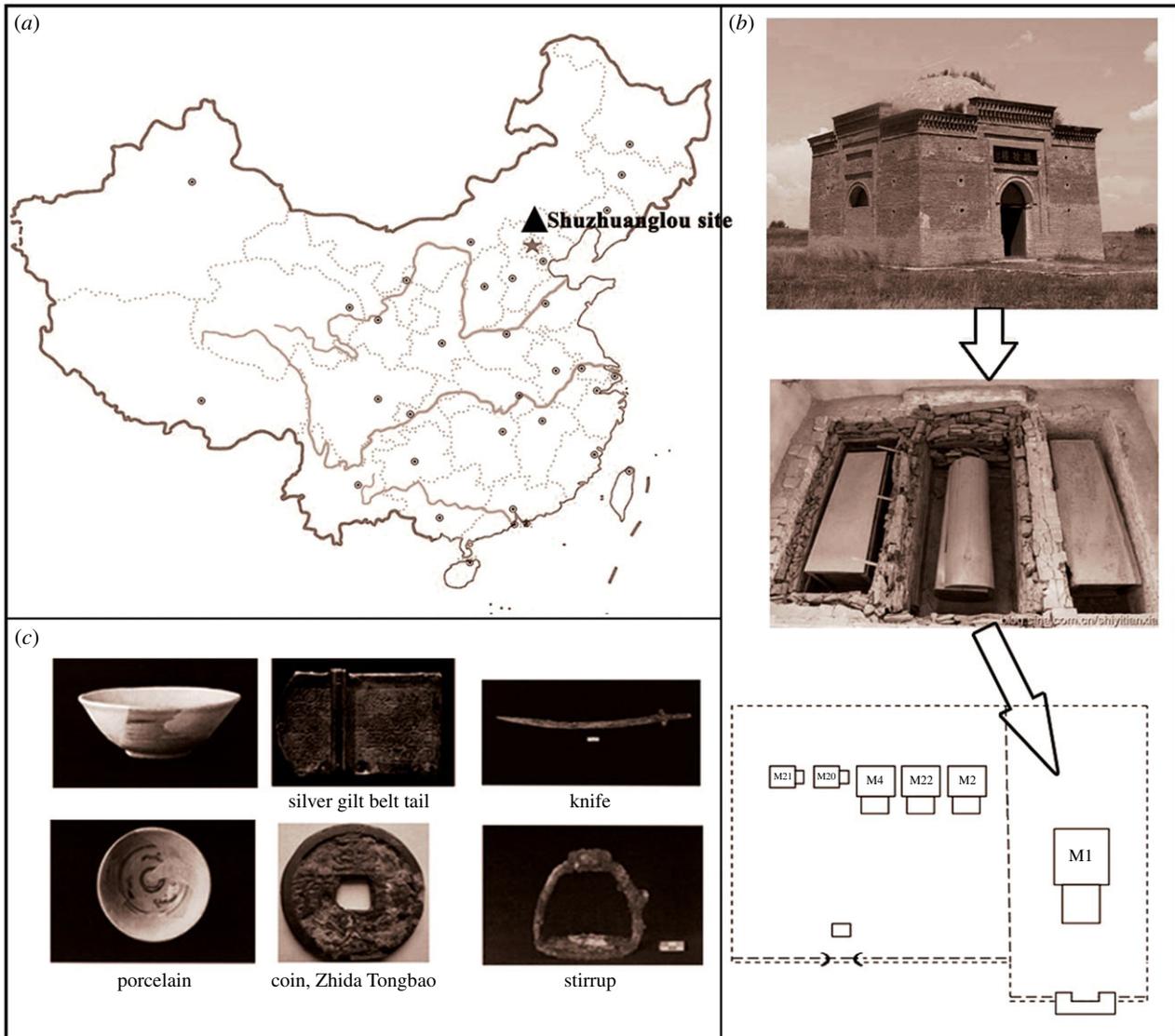
The Yuan Dynasty (AD 1271–1368) was the first dynasty in Chinese history where a minority ethnic group (Mongols) ruled. Few cemeteries containing Mongolian nobles have been found owing to their tradition of keeping burial grounds secret and their lack of historical records. Archaeological excavations at the Shuzhuanglou site in the Hebei province of China led to the discovery of 13 skeletons in six separate tombs. The style of the artefacts and burials indicate the cemetery occupants were Mongol nobles. However, the origin, relationships and status of the chief occupant (M1m) are unclear. To shed light on the identity of the principal occupant and resolve the kin relationships between individuals, a multidisciplinary approach was adopted, combining archaeological information, stable isotope data and molecular genetic data. Analysis of autosomal, mitochondrial and Y-chromosomal DNA show that some of the occupants were related. The available evidence strongly suggests that the principal occupant may have been the Mongol noble Korguz. Our study demonstrates the power of a multidisciplinary approach in elucidating information about the inhabitants of ancient historical sites.

## 1. Introduction

### (a) Archaeological background of the Yuan cemetery

In Chinese history, the Yuan Dynasty (1271–1368) was the first dynasty established by a minority ethnic group (Mongols). Owing to the tradition of keeping ancestral burial grounds secret and the absence of historical records, few burial sites of the Mongols of the Yuan Dynasty have been discovered, especially those of Mongolian nobles [1]. Archaeologists and historians are interested in the unique burial customs and kinship organization of high status Mongols, which can help clarify the characteristic culture and social structure of the Yuan Dynasty.

A well-preserved ancient building named Shuzhuanglou, known as the palace of the Queen of the Liao Dynasty (916–1125), is located in the northeast region of Guyuan county, Hebei province (figure 1*a,b*). In 2000, archaeologists accidentally discovered an intact tomb under the building. The tomb was divided into three chambers: the central chamber contained a male skeleton



**Figure 1.** (a) The location of the Shuzhuanglou site; (b) top: the building above ground, and the tomb (M1) under the building, bottom: map of the cemetery; (c) some of the artefacts recovered from the Shuzhuanglou site. (Online version in colour.)

buried in a tree-coffin, and the two adjacent side chambers contained female skeletons without tree-coffins. The tree-coffin was made using a whole tree trunk, carving space for a human shape out of the middle and binding it with an iron hoop. According to official records, only noble Mongols had the right to use tree-coffins for burial [2]. The tree-coffin contained a silver belt with a dragon pattern, coins and Mongolian dress items, further confirming the tomb occupant was probably a Mongolian noble. Five more slightly smaller tombs of the same style were situated in a row to the west of the principal tomb, and all of them contained one male and one female (figure 1b). Historians and archaeologists have suggested the whole cemetery belongs to a Mongolian noble or royal family, and the building above ground was a sacrificial hall, rather than the palace of the Queen of the Liao Dynasty. However, the style of the building, such as the arched dome, is more typical of the styles of northwest China than those of the Mongols, and this raises questions about the identity of the tomb occupant and the relationship between the burials [3]. The cemetery provides a unique opportunity to investigate the burial customs and kinship organization of high status Mongols from the Yuan dynasty.

### (b) Analysis using a multidisciplinary approach

Advances in the analysis of human archaeological remains, including the use of ancient DNA and stable isotope analyses, can offer valuable tools for archaeological investigations. Genetic analyses of the uniparentally inherited mitochondrial DNA (mtDNA) and Y-chromosome single-nucleotide polymorphisms (SNPs), as well as autosomal short tandem repeats (STRs) from human samples can help determine the sex of individuals and kinship relationships [4–6], as well as help identify historical figures [7]. Stable isotope ratios of oxygen and hydrogen in human tissues can vary owing to differences in diet and drinking water and are thus informative on human mobility patterns [8]. Using these approaches, we can obtain detailed genetic and diachronic isotopic information and reconstruct aspects of individual identity and social background. For example, Haak *et al.* [9] gained detailed insight into familial relationships and the marriage system in Central Europe during the Late Stone Age by using a combination of ancient DNA and strontium isotope analyses.

The human remains investigated in this study consist of 13 skeletons in six separate tombs from the Shuzhuanglou site. A combined approach using archaeological, anthropological, stable isotope and ancient DNA methods was applied

to identify the chief occupant of the cemetery and resolve the familial relationships.

## 2. Material and methods

### (a) Samples

The samples were from a presumed Mongolian noble cemetery in the Shuzhuanglou site at Guyuan County, Hebei Province, China, which dates to the Yuan Dynasty according to archaeological evidence [3]. The burial site, originally surrounded by a periphery wall, was divided into two parts by brick walls: the main grave (M1), and the five smaller tombs (M2, M4, M20, M21 and M22) are on the western side (figure 1*b*).

Teeth and/or skeletal samples of all 13 individuals in the six tombs were collected for ancient DNA and isotope analyses (table 1). For most individuals, intact teeth without cracks were available, and a limb bone was collected if no intact teeth were available. Water had seeped through some of the burials, and the skeleton M4w was so heavily decayed no suitable sample could be chosen. Age of death was estimated by dental formation, skeletal ossification and circumference of the cranium, and shown in the electronic supplementary material, table S1.

### (b) Archaeological and anthropological findings

All the tombs are rectangular, with the individuals lying in a north to south orientation. Wooden coffins in all tombs followed a pattern of one coffin plus one outer coffin ('guo, 椁'), except in the tombs of M2w and M21e, where only a single coffin was found in each. The wooden coffins in the tombs of M1m and M20e were tree-coffins.

The cemetery had been looted at an earlier date, so only a few artefacts remain (electronic supplementary material, table S1, and figure 1*c*). These include a gold earring, pieces of a brooch, a silver belt with a dragon pattern, black-glazed porcelain and a Gugu hat—an ornament only worn by Mongolian noblewomen. In the M1, M2 and M21 tombs, several coins were found, most of them dating to the Tang (618–907) and Song Dynasties (960–1127). A coin (Zhida tongbao) from the Yuan Dynasty, dated AD 1310–1311, was discovered in the tomb of M1m, which limits the time of the burial to after 1310. A piece of stele with the Chinese characters '阔里吉斯, korguz,' was found in the burial chamber of M1m, and it is thought to carry the name of the tomb occupant. The burial format and unearthed relics demonstrate the high rank of the tomb occupants. It is worth noting that the female burials contained more artefacts than those of the males in each of the joint burial tombs, except in tombs M1 and M21.

### (c) Methods to avoid DNA contamination and monitor authenticity

Appropriate precautions were taken to ensure the authenticity of the ancient DNA results. All pre-polymerase chain reaction (PCR) steps were performed in a positive pressure laboratory dedicated to ancient DNA located in the Research Centre for Chinese Frontier Archaeology of Jilin University. Different rooms were used for sample preparation, DNA extraction and setting up PCR. Post-PCR procedures were carried out in a different building. Surfaces were cleaned regularly with a 10% sodium hypochlorite solution and irradiated with ultraviolet (UV) light (254 nm), and full-body protective clothing, facemasks and gloves were worn. Gloves were changed frequently. All consumables were purchased as DNA-free, while reagents were irradiated with UV light for at least 20 min. Every PCR assay included extraction and amplification controls. To check for reproducibility, the experiments were duplicated using another sample from each individual in the Molecular Forensic Laboratory in the

School of Life Sciences of Jilin University. At least two PCR amplifications per SNP were done in each laboratory. To identify potential contamination from laboratory personnel, the mtDNA and STR profiles of all staff in the project were obtained.

### (d) Ancient DNA analysis

#### (i) Ancient DNA extraction

Teeth samples were treated before DNA extraction as described by Li *et al.* [10]. Tooth powder (0.1 g) was incubated for 24 h in 3 ml of a solution containing 0.45 M EDTA, 0.5% SDS and 0.7 mg ml<sup>-1</sup> proteinase K in a shaker (220 r.p.m.) at 50°C. Afterwards, DNA was extracted using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

#### (ii) Mitochondrial DNA analysis

Two sets of overlapping primers were used to amplify the mtDNA hypervariable segment I (HVS-I) region between positions 16035 and 16409, and PCR amplifications were done for all the ancient samples as described by Li *et al.* [10], but increasing the number of cycles to 40. Amplification products were sequenced directly using the ABI 310 Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequence reaction products were analysed on an ABI PRISM 310 automated DNA sequencer. To confirm the mtDNA haplogroups, selected SNPs of the mitochondrial coding regions were typed. The PCR reaction conditions were the same as those for the mtDNA HVS-I amplification.

The mitochondrial genome of the chief occupant (M1m) was sequenced on an Illumina HiSeq2000 platform. Two separate libraries were prepared from the same ancient DNA extract of 30 µl following the Illumina TruSeq DNA sample preparation protocol, except that a 1 : 10 diluted adapter was applied to the ends of DNA fragments during ligation. The construction of libraries and PCR set-up for amplification of libraries were carried out in a PCR hood at the ancient DNA laboratory, while PCR amplification was carried out in thermo cyclers located in a laboratory for contemporary DNA experiments, where libraries were amplified for 15 cycles and then followed by size-fractionation on a 15% acrylamide gel to entirely remove adapter dimers. The quality and concentration of the two libraries were determined on an Agilent Bioanalyzer 2100 and multiplex shotgun sequencing was carried out using an Illumina HiSeq2000 platform at the high-throughput DNA sequencing centre of Peking University. One hundred and one base pair (bp) paired-end reads and a single index read were generated according to manufacturer's instructions.

#### (iii) Molecular sex determination

Fragments of the amelogenin gene (AMEL) were amplified in a PCR reaction with fluorescent-labelled primers as described by Baca *et al.* [11]. The PCR reaction conditions were the same as those for the mitochondrial HVS-I amplification. PCR products were analysed on an ABI Prism 310 Genetic Analyzer and GENEMAPPER software v. 4.2 (Applied Biosystems). At least two independent PCR reactions were performed for each DNA sample.

#### (iv) Y-chromosome single-nucleotide polymorphism analysis

Taking into account the status of Mongol nobles inferred from archaeological information, seven biallelic markers that characterize the main lineages in modern and ancient Mongolian populations (C, N, O and Q) were tested using a hierarchical genotyping strategy [12]. First, the five Y-chromosome markers C-M216, F-M89, K-M9, P-M45 and NO-M214 were genotyped. Afterwards, the P-M45-derived individuals were subjected to further typing of markers Q-M242 and R-M173. PCR amplification primers and procedures were carried out as described by Li *et al.* [10]. The length of the PCR amplicons was typically between 100 and 200 bp.

**Table 1.** Polymorphisms of mtDNA and Y-chromosome SNPs in the 13 Shuzhuanglou individuals. (HVS-I, hypervariable segment I; SNPs, single-nucleotide polymorphisms. The meaning of sample's name: M means tomb, number was the order no. of the whole cemetery, and the small letter m, e and w means individual's orientation in the tomb, and each represent as middle, east and west. A dash means data unavailable.)

tomb	sample no.	morphological	molecular	mtDNA HVS-I (minus 16000)	mtDNA-coding SNPs	mtDNA haplogroup	Y SNPs	Y haplogroup
M1	M1m	male	male	214, 223, 362	+10397 <i>AluI</i> , -5176 <i>AluI</i>	D4m2	M89C > T, M9C > G, M45G > A, M242 C > T	Q
M2	M1e	female	female	129, 183, 319, 362	+10397 <i>AluI</i> , +663 <i>Hae III</i>	A		
	M1w	female	—	—	—	—		
	M2e	female	female	—	+10397 <i>AluI</i> , -5176 <i>AluI</i>	D		
M4	M2w	male	male	136, 189, 300	9 bp del	B		
	M4e	female	female	214, 223, 362	+10397 <i>AluI</i> , -5176 <i>AluI</i>	D		
	M4w	male	—	—	—	—		
M20	M20e	female	female	129, 183, 319, 362	+10397 <i>AluI</i> , +663 <i>Hae III</i>	A		
	M20w	male	male	223, 290, 319	+10397 <i>AluI</i> , +663 <i>Hae III</i>	A	M89C > T, M9C > G, M45G > A, M242 C > T	Q
M21	M21e	female	female	189, 223, 362	+10397 <i>AluI</i> , -5176 <i>AluI</i>	D		
	M21w	male	male	223, 290, 319	+10397 <i>AluI</i> , +663 <i>Hae III</i>	A	M89C > T, M9C > G, M45G > A, M242 C > T	Q
M22	M22e	female	female	223, 266, 290, 311	+10397 <i>AluI</i>	—		
	M22w	male	—	—	—	—		

**Table 2.** Sample information and contents of H, and O isotopic data.

sample location	collagen yield (%)	$\delta^{18}\text{O}$	$\delta\text{D}$
M1m	4.7	19.3	-142.4
M1e	3.6	6.9	-49.1
M1w	5.7	7.9	-41.8
M4w	3.8	7.3	-46.1
M20w	4.9	7.2	-44.3
M20e	4.4	7.1	-38.7
M21e	5.7	8.0	-40.6
M21w	3.8	7.9	-46.6

### (v) Autosomal short tandem repeat analysis

Autosomal STR analysis of the ancient samples was performed on nine loci using the AGCU mini STR Kit (AGCU ScienTech, China). Experimental conditions were as recommended by the manufacturer, but the number of PCR cycles was increased to 40. STR products were analysed on an ABI Prism 310 Genetic Analyzer with GENEMAPPER software v. 4.2. In order to establish the kinship relationships among different individuals, the cumulative paternity index (CPI) and the probability of paternity ( $W$ ) were calculated [13]. All calculations were based on allelic frequencies estimated for northern Chinese populations [14].

### (e) Stable isotope analysis

#### (i) Collagen extraction

Collagen was extracted from the human bone samples using a modification of the protocol of Jay *et al.* [15]. For each individual, 2 g of bone sample was mechanically cleaned to remove outer and inner contaminants, and then demineralized in 0.5 M HCl at 4°C. The liquid was changed every 2 days until the samples were soft and bubbling ceased. The remaining residue was washed with deionized water until the pH returned to neutral, and then the residue was rinsed in 0.125 M NaOH for 20 h at 4°C and washed again with deionized water. Afterwards, the remains were rinsed in 0.001 M HCl, gelatinized at 70°C for 48 h and filtered. The residues were freeze-dried for 48 h to recover the collagen. The collagen yield was calculated by dividing the dried collagen weight by the bone sample weight (table 2).

#### (ii) Stable isotope measurements

The collagen samples and standards for isotopic measurements were placed in the open at room temperature for 48 h to remove the influence of the exchanged hydrogen from the surrounding air. Thus, the  $\delta\text{D}$  values were measured under the same conditions to enable a direct comparison of the samples. The samples were pyrolyzed at high temperature, and the H and O isotope values measured in an Isoprime-100 IRMS coupled with an Elementary Pyrocube system. The standards for measuring the H and O isotopes were IAEA-601 and IAEA-CH7, respectively. The precisions were 1.5 and 0.3‰. The stable isotope values for H and O are expressed by  $\delta\text{D}$  and  $\delta^{18}\text{O}$  relative to standard mean ocean water.

## 3. Results and discussion

### (a) Authenticity of the ancient DNA results

Strict procedures were used to prevent modern DNA contamination. We regard our results as authentic based on a

number of different observations: (i) the negative extraction and amplification controls were always free of contamination; (ii) the results were repeatable and reproducible, as verified by performing at least two duplicated extractions, and two duplicated amplifications of each extract; (iii) the Y-SNP and autosomal STR profiles of the ancient individuals were different from those of the laboratory researchers; and (iv) we observed an inverse relationship between amplification efficiency and the size of the autosomal STRs.

### (b) Sex determination

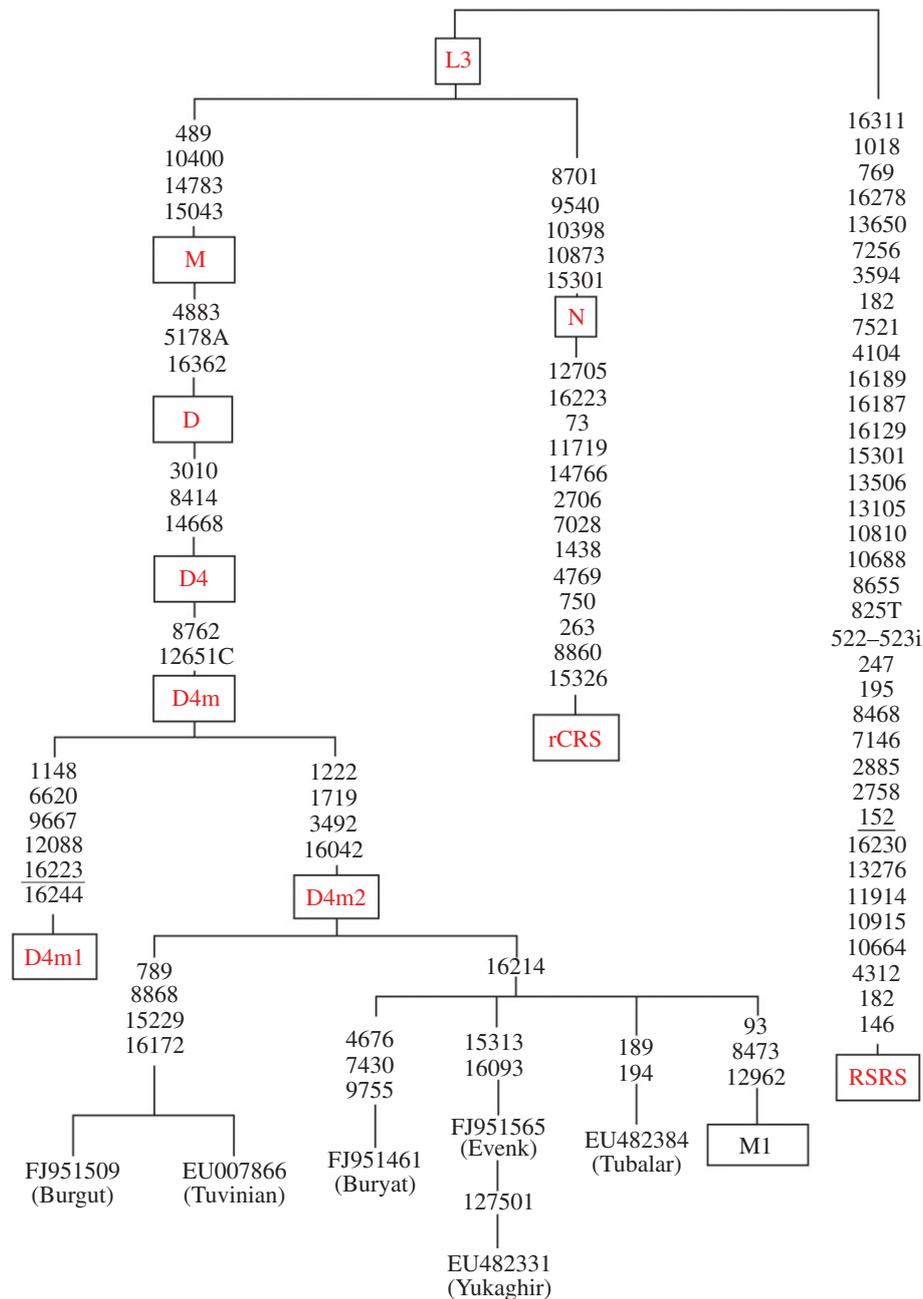
As shown in table 1, amplification of the AMEL gene was successful in all the individuals except M1w, M4w and M22w. Molecular and anthropological sex determinations were compared, and genetic data matched anthropological determinations. The sex determination results indicate that all the tombs on the west side were joint burials, in which a man was placed in the west part of the tomb while a woman was placed in the east part. In the M1 tomb, three individuals were buried together; in the middle, there was a male, and the two females were buried on each side of him.

### (c) Ancient DNA analysis

Reproducible sequences were obtained for nine out of 13 individuals (table 1). The 393 bp fragment of the mtDNA HVS-I was compared with the revised Cambridge Reference Sequence [16]. There were 12 polymorphic sites, including 11 transitions and one transversion (16183A-C). Using the HVS-I and coding region data combined with the eastern Eurasian mtDNA classification tree [17,18], the nine sequences, representing six haplotypes, were assigned to three haplogroups (table 1). The main haplogroups in the Shuzhuanglou people were A and D, each shared by four individuals. Haplogroups A and D have high-frequency distributions in the ancient and modern Northern and Eastern Asian populations. The remaining haplogroup was B, which was found in only one Shuzhuanglou individual (M2w). Individual pairs M1m and M4e, M20w and M21w, and M1e and M20e, shared the same mtDNA profile, suggesting they were maternally related.

For the six males in the cemetery, only half yielded Y-chromosome SNPs. Three (M1m, M20w and M21w) exhibited the same SNP motif F-M89, K-M9, P-M45 and Q-M242, attributed to haplogroup Q. Tombs M20 and M21 are adjacent, and both have a dais oriented on the east west axis (figure 1b), while tombs M1, M2, M4 and M22 all had a dais with a southern orientation. Our ancient DNA results show that the two males from M20 and M21 (M20w and M21w) had the same maternal and paternal type, suggesting they could be brothers, but we cannot rule out the possibility that they were cousins, or uncle and nephew.

All ancient samples were analysed at nine autosomal STR loci. Four of the 13 individuals yielded results for at least five loci in two independent extractions. Consensus data are reported in table 3. The autosomal STR genotyping results show that the M2w and M4e DNA profiles shared one allele in each of the investigated loci. As they had different mtDNA profiles, the STR data suggest a possible paternity kinship between M2w (father) and M4e (daughter) (99.672 and 99.007% for CPI and  $W$ , respectively). The man and woman in tomb M21 had different mtDNA and STR profiles, meaning they were unrelated, which confirms the view that the two individuals sharing a tomb could have been husband and wife.



**Figure 2.** Phylogeny of the complete mitochondrial genome of M1m, which was sequenced in this study. (Online version in colour.)

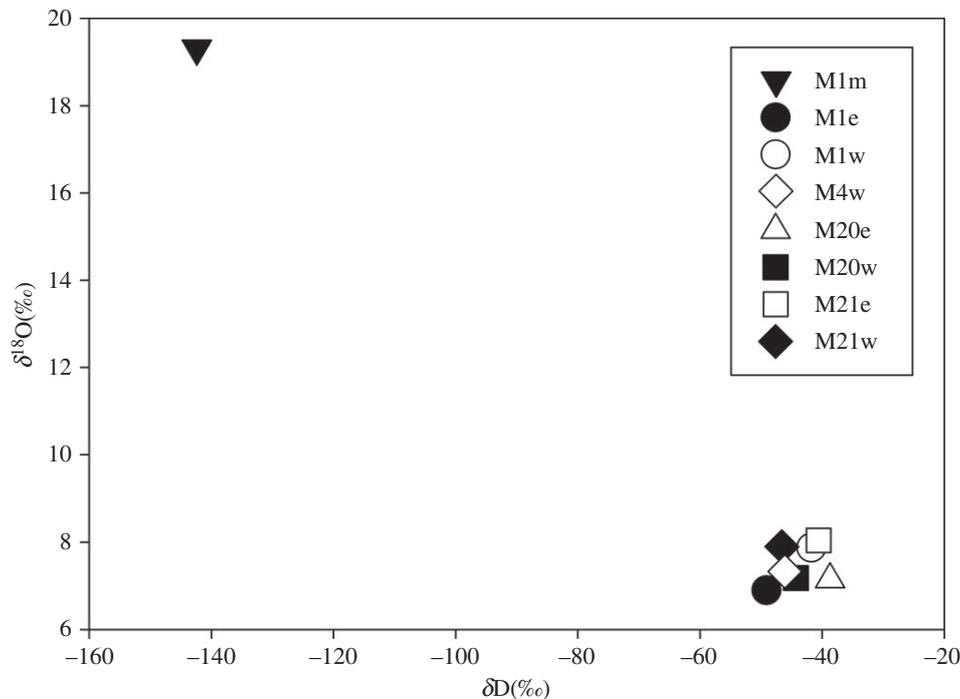
**Table 3.** Autosomal STR genotypes of the four specimens from the Shuzhuanglou site. (To be typed unequivocally as homozygous for a given locus, at least three amplifications must show consistent reproduction of the same allele. Allele products were detected in at least two amplifications. In some cases, determination was not possible: this is signified by a dash.)

	Amel	Penda E	D12S391	D6S1043	D2S1338	D19S433	CSF1P0	PendaD	D19S253
M2w	X/Y	13/17	19	18/21	18/20	14	11	9/10	-/-
M4e	X	13/16	17/19	11/18	20/24	14/16	10/11	9/12	12
M21w	X/Y	11/14	18/21	11/18	20/27	14/15	10/12	9	12/14
M21e	X	12/-	18/-	10/13	23/24	16/-	10/11	12	-/-

Thus, the genetic data reveal family ties among individuals in the cemetery, both through maternal and paternal lines, confirming the archaeological hypothesis that Shuzhuanglou was a family cemetery.

To help determine the identity of individual M1m, the complete mitochondrial genome was sequenced. Although the

fraction of endogenous DNA was below 1%, a nearly complete mitochondrial genome with about 10× coverage was obtained from 4 gigabases (Gb) of two combined libraries' shotgun sequences. SNPs and INDELs were identified using Genome Analysis Toolkit [19] after removal of PCR duplications by SAMTOOLS v. 0.1.17 [20]. Mitochondrial haplogroups and



**Figure 3.** Scatter plot of H and O isotope values of samples from the Shuzhuanglou site.

haplotypes were assigned with the software HAPLOGREP (<http://haplogrep.uibk.ac.at>) [21], and the highest score was selected based on the phylogenetic software, TREEBUILD v. 15 [22] (<http://www.phylotree.org>). M1m was assigned to haplogroup D4m2 based on variants in the mitochondrial genome (figure 2). The same haplogroup and substitutions (via restriction fragment length polymorphism and HVS-I sequence) were confirmed by Sanger sequencing. Haplogroup D4m, a rare branch of haplogroup D, has a very distinctive geographical distribution: the subtype D4m1 is only found in Japan and the other subtype D4m2 is mainly found in central and southern Siberia [23]. This suggests that M1m originated from the nomads of the North Eurasian steppe.

The Y-chromosome haplogroup of M1m is Q, a haplogroup found at low frequencies throughout the Middle East, Asia and Siberia, and at high frequencies in the Americas. Y-chromosome analysis of ancient and modern Mongolian populations has shown that the most prevalent Y haplogroups are C, N and O [24–26]. However, the Y haplogroup attributed to the Mongolian emperor Genghis Khan and his descendants, the so-called Golden Family, is much debated. Some studies suggested that the male descendants of Genghis Khan belong to group C3\* [25,26]. Batbayar and colleagues tested direct descendants of Genghis Khan and showed that the three remaining lineages of Genghis Khan have three different subgroups of haplogroup C3 [27]. It is generally agreed upon that the Y-chromosome lineage of the royal family of Mongolia, the Golden Family, belongs to haplogroup C3. If this is true, the males at the Shuzhuanglou site were not part of the Golden Family, but instead belonged to other Mongol noble families or northern steppe tribes.

#### (d) Hydrogen and oxygen isotope analysis

The H and O isotope values in organisms are determined by their diet and drinking water, which derive directly from the environment. Thus, analysis of these isotopes along with carbon and nitrogen isotopes can provide important information on climate, environment and migrations. The individuals in

figure 3 can be divided into two groups according to  $\delta D$  and  $\delta^{18}O$  values. The first group includes M1e, M1w, M20e, M20w, M21e, M21w and M4w, with mean  $\delta D$  and  $\delta^{18}O$  values of  $-43.9 \pm 3.7\text{‰}$  ( $n = 7$ ) and  $7.5 \pm 0.3\text{‰}$  ( $n = 7$ ). Compared to the above, the M1m sample has abnormal  $\delta D$  ( $-142.4\text{‰}$ ) and  $\delta^{18}O$  ( $19.3\text{‰}$ ) values, strongly suggesting that he originated somewhere else and moved to Hebei before his death.

#### (e) Synthesis of different data

The available archaeological and anthropological information, combined with the stable isotope and ancient DNA data, suggests the cemetery was occupied by a high status family of the middle period of the Yuan Dynasty. The chief occupant (M1m) was a male, aged about 40 years (based on anthropological data), and most likely of North Eurasian nomad ancestry (based on the presence of mtDNA haplogroup D4m2 and Y haplogroup Q). He was of high social status (based on the material goods in the grave) but he was not a member of the Golden Family (based on the presence of Y haplogroup Q). Based on the isotope results, the man was away from his family for long periods of time, and he was buried after 1310 (based on the date of the coin).

Combining the above conclusions with the clue of a name on the broken stele, a search through the available historical record leads to one promising candidate, Gaotang Wang, Korguz, the fifth chief of the Ongüid tribe and the descendant of the ancient Turki (tujue, 突厥), who were good allies of Kublai Khan. According to the historical record [28], Korguz married Kublai's two granddaughters and fought against Kaidu, whose protégé, Duwa, captured and killed him in 1298. His son reburied him in 1311. The two females in the M1 grave might be two Mongolian princesses. Korguz spent most of his lifetime on the northwest frontier, which could explain the difference in his O/H isotope values. Mongolian rulers have a tradition of bestowing their daughters to lords of Ongüid to forge military alliances. According to historical

records, eight Mongolian princesses were married to lords of Ongüd during the generation of Korguz [28], which may explain the higher status of the female tombs relative to the male tombs.

There is speculation about the identity of the tomb occupant, and other figures have been proposed [3,29], such as Anxi Wang Ananda, Kublai's grandson and another Korguz living at the end of the Yuan Dynasty. Ananda, a descendant of the Golden Family, was killed for usurping the throne in 1307, and the Y-chromosome haplogroup and burial time based on the coin rule out this possibility. As for the other Korguz, he spent his entire life serving at the court of the Yuan Dynasty. The evidence from the stable isotope analysis thus suggests that he is an unlikely candidate. In conclusion,

our genetic data demonstrate that the cemetery might contain closely related individuals, and possible familial relationships can be proposed. Combining the historical record, archaeological information, isotopes and ancient DNA analyses, we can address the identity of the human remains. This case study demonstrates that such a multidisciplinary approach is an efficient method for individual and kinship identification and for revealing novel information about past societies.

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