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SHORT COMMUNICATION

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Genetic analysis of human remains from a double inhumation in a frozen kurgan in Kazakhstan (Berel site, Early 3rd Century BC)

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Abstract The discovery of a big barrow of the Saka period in eastern Kazakhstan between the Russian and the Chinese borders provided the opportunity to excavate a frozen burial site. In the burial chamber, there was a wooden sarcophagus with two human bodies. The skeletons of these two individuals, a man and a woman, were well preserved. A genetic study based on STRs and mitochondrial DNA analyses was undertaken in order to determine whether these human remains belonged to close relatives. Results were obtained for all the markers. Nevertheless, nuclear STRs did not allow a clear conclusion concerning the relationship, but analysis of mitochondrial DNA showed that these skeletons were not close relatives.

Keywords Ancient DNA · Mitochondrial DNA · STRs · Frozen bone samples · Central Asia

Introduction

In 1997 an international archaeological expedition managed by Z. Samashev and H.P. Francfort discovered a frozen Scythian grave under a stone tumulus (kurgan) near the village of Berel in the Bukhtarma valley (east Kazakhstan), between the Russian and the Chinese borders. This grave belonged to a Saka Prince and could be dated back to the early third century BC. In this region where winters are usually very cold, several burial places located under kurgans have been discovered, protected by ice formed just after inhumation. This kurgan is similar to those located in the Ukok high plateau (Russia).

Scythians were an Indo-European people who lived in the Kazakhstan steppes (700 BC–200 AD). The history of the Scythians and Sakas is only known from narrations of Herodotus (480–425 BC) and by the excavation of their burial places.

Concerning the grave discovered in 1997, the Saka prince was buried in a wooden sarcophagus. The funeral chamber was 6 m deep and divided in two parts, the first containing the sarcophagus and the second containing well preserved remains of 13 horses sacrificed along with their harnesses. Only a small part of the funeral chamber had avoided being sacked, but the sarcophagus had been broken. The body was totally stripped, but without skeleton perturbation: the anthropologists discovered all the bones still connected. Anthropomorphic analyses suggested that these remains belonged to a male 30–50 years old (subject 1).

Near this body, in the same sarcophagus, a second less well preserved body was discovered during the excavation. Anthropologists thought that these remains probably belonged to an old woman (subject 2). During the excavation, it could be proved that the woman had been buried after the man and many years after the sacking of the sarcophagus.

According to the archaeologists, double burial was an unusual practice at this time and DNA analysis was performed in order to determine whether these human remains did or did not belong to close relatives. A genetic approach may be an interesting tool for anthropologists and archaeologists in understanding funeral practices in those times.

Materials and methods

Materials

The two bodies discovered in the frozen grave were well preserved: some part of muscles, brain and hair were still present. The

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viscera and the soft tissues of the man had been removed before inhumation. The woman had been buried with no post-mortem modifications.

Genetic analyses were performed on different biological samples: bone, brain, and soft tissues (muscles) from each individual. Brain samples and soft tissues were very altered and bone samples were covered with a black-brown sediment probably due to bacterial degradation. Samples were frozen during the excavation and stored at –20°C.

Methods

DNA extraction

The bone samples were cleaned and abraded with a sterile scalpel blade and powdered in a 6800 freezer mill (Fischer Bioblock) in liquid nitrogen.

DNA extraction was made according to Fily et al. 1998. Briefly, 2 g of the sample (bone powder or tissues) was used to extract DNA after a 16 h incubation at 56°C in an extraction buffer (5 mM EDTA, 2% SDS, 10 mM Tris HCl pH 8.0, 0.3 M sodium acetate, 1 mg/ml proteinase K). After an organic extraction (phenol/chloroform/isoamyl alcohol, 25:24:1, v/v/v), the aqueous phase was purified with the help of the CleanMix kit (Talent, France) a method based on the DNA affinity for the silica in the presence of guanidine thiocyanate. Samples were concentrated to 40 µl using Microcon-30 filters (Millipore, France).

STR analysis

Amplifications were performed with the AmplF/STR Profiler Plus kit (PE Applied Biosystems) which allows simultaneous amplification of nine STRs (D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) and the amelogenin locus.

PCR conditions were modified compared to the manufacturer conditions as follows: PCR reaction mix 3.82 µl, primer set 2 µl, AmpliTaq Gold 0.182 µl and 1–4 µl of DNA sample to a final volume of 10 µl and 37 cycles of PCR were necessary to amplify these samples.

Second STR amplifications were performed with the AmplF/STR SGM plus kit (PE Applied Biosystems) which allows to amplify simultaneously ten Loci (D3S1358, vWA, FGA, D21S11, D8S1179, D18S51, D16S539, D2S1338, D19S433, TH01) and the amelogenin locus too.

PCR conditions used were as described in the manufacturer's manual.

PCR products were analysed on automatic sequencers, the 373ABI (PE Applied Biosystems) (on 6% acrylamide gels, Sequagel 6%) and the 310ABI (PE Applied Biosystems).

Mitochondrial DNA analysis

Mitochondrial DNA (mtDNA) analyses were performed on the first hypervariable segment of the control region (HV1) divided in two sub-regions (a and b) respectively with the primers:

- L15990 5'-TTAACTCCACCATTAGCACC-3' (Ivanov et al. 1996)
- H16239 5'-TGGCTTTGGAGTTGCAGTTG-3' (Ivanov et al. 1996)
- L16159 5'-TACTTGACCACCTGTAGTAC-3' (Fisher et al. 1993)
- H16401 5'-TGATTTCACGGAGGATGGTG-3' (Vigilant et al. 1989).

Amplifications were performed with 2–6 µl of the DNA extract, 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mg/ml BSA, 200 µM each dNTP, 0.25 µM each primer and 2 U of Taq Gold Star (Eurogentec, Belgium). Amplification conditions were: denaturation for 10 min at 94°C followed by 40 cycles of 45 s at 94°C, 1 min at 60°C for HVIa or 53°C for HVIb, elongation for 2 min at 72°C, extension 10 min at 72°C and storage at 4°C.

Amplification products were checked on a 1% agarose gel and purified with Microcon-PCR filters (Millipore, France). Sequence PCR was performed with the ABI Prism Dye Terminator Ready Reaction kit (PE Applied Biosystems) according to the manufacturers conditions and analysed on the 373ABI automatic sequencer (PE Applied Biosystems) (on 6% acrylamide gel, Sequagel 6%).

Precautions against contamination

During all the steps of the handling of the samples, all precautions needed against contamination for analyses of DNA extracted from ancient tissues were taken. The samples were always handled with gloves including during excavation, and frozen on the site of the fieldwork. Material used for the analysis was sterilised by autoclave and/or long UV exposure (laminar flux hood, laboratory coats, pipettes, filter tips, tubes, filters and tubes from kits). Negative controls were performed throughout the extraction and PCR procedures. In order to detect possible exogen sources of contamination, haplotypes and mitotypes of all the anthropologists and laboratory workers were defined from blood samples and were compared to our results. In order to limit cross-contamination, the samples were not in contact with other DNA samples during all the experimentation.

At least five extractions and three amplifications on different extractions were made for each sample to assess the reproducibility and the authenticity of our results.

Results

Amplifications were successful from bone samples only. The other samples showed a high level of PCR inhibition. Due to the good preservation of bone samples, reproductive results were obtained from at least three different extractions for both individuals.

STR analysis

The results are shown in Table 1 and electropherograms for the SGM Plus amplification are shown in Fig. 1.

Table 1 STR results for the subjects 1 and 2 from bone samples with the Profiler Plus and SGM Plus kits

STR System	Subject 1	Subject 2
Amelogenin	XY	XX
D3S1358	14/15	15/15
vWA	16/17	16/16
FGA	22/22	22/23
D21S11	29/29	30/30
D8S1179	11/13	13/13
D18S51	19/23	13/17
D5S818	11/13	11/12
D13S317	10/11	11/11
D7S820	10/11	8/11
D16S539	9/11	10/12
D2S1338	19/22	23/23
D19S433	12/13	13/13
TH01	9.3/9.3	7/9

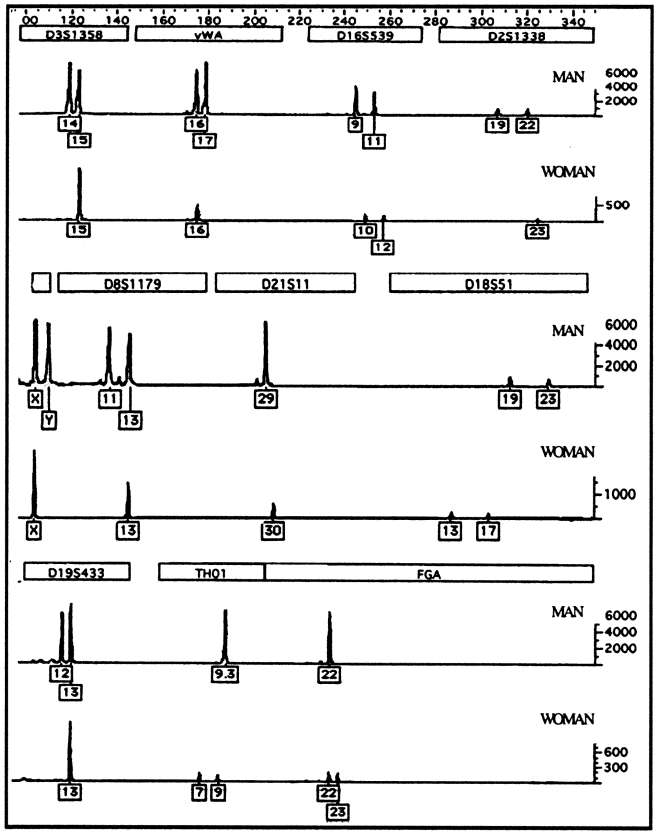

Fig. 1 Electrophoregrams obtained with the SGM Plus amplification kit for the two DNA samples from skeletons buried together in the kurgan

The amplification of the amelogenin locus allowed us to determine that sample 1 was a man and sample 2 was a woman.

Results were obtained for all the eight STR loci with the Profiler Plus kit. We observed some ambiguities in the locus vWA for which the smaller allele was not so strongly amplified as the larger one. With the SGM Plus kit all loci were amplified without uncertainties.

Mitochondrial DNA analysis

Sequences obtained from the two samples were compared to each other and to the reference sequence (Anderson et al. 1981) (Table 2).

Table 2 Positions of the base changes of subjects 1 and 2 for the mitochondrial region HVI analyse between the bases 16021 and 16417

	Base position		
	16223	16278	16362
Anderson	C	C	T
Subject 1	C	C	T
Subject 2	T	T	C

Discussion

The objective of this study was to amplify genetic markers on bone samples more than 2000 years old, and to show a possible kinship between the 2 subjects buried together. Several studies have already shown the importance of the autosomal STR typing by multiplexing for degraded DNA samples (DNA amounts reduced for the analysis, possible detection and determination of the origin of contamination) (Hummel et al. 2000; Schultes et al. 2000). For our study, numerous genetic markers were amplified, a total of 13 STRs and the complete HV1 region of the mitochondrial DNA. The results obtained for the STR analysis of the two individuals were compared with the genotypes of all the personnel involved. Moreover, their sequences were compared to the mitotypes of the technicians and archaeologists. All were different. The reproducibility of our results and all the precautions taken against contamination are relevant with authentic results obtained from ancient bone tissues.

The amplification of the amelogenin locus was consistent with the morphological sexual determination that subject 1 was a man and subject 2 a woman. This genetic confirmation of the sexual morphological determination is another proof of the authenticity of the DNA samples studied.

13 STR loci were amplified. With the Profiler Plus kit, some doubts remained especially for sample 2. Except for the microsatellite D18S51 without doubt, the two samples had one allele in common for each locus. The genotypes of the subjects that could be determined for eight STRs revealed that they could be closely related such as a mother-son, a father-daughter or a brother-sister kinship.

The use of the SGM Plus kit clarified the results: no uncertainty was detected, the alleles for the common markers with Profiler Plus were confirmed and the alleles obtained for the additional loci showed differences between the two subjects. Therefore, a relationship such as mother-son or father-daughter could be excluded, but according to these results, a brother and sister kinship was still possible.

The mtDNA analysis showed three mutation points between the sequences obtained for the HV1 region for samples 1 and 2 after a comparison between them and the Cambridge sequence (Anderson et al. 1981). Thus, these DNA samples could not come from a brother and his sister or from people related by a maternal lineage.

These results show that the two bodies discovered in the frozen grave in the Berel site were not close relatives. Nevertheless, this double inhumation could have been set up for a man and his wife.

The good preservation of the remains discovered in the kurgan and the good quality and quantity of the DNA extracted from the bones can probably be explained by the frozen state of the grave.

The HV1 mitochondrial sequence of subject 1 was similar to the Anderson sequence. In comparison with present day populations, its frequency is 16% in a combined set

of 401 European individuals (Baasner and Madea 2000; Crespillo et al. 2000; Piercy et al. 1993; Tagliabracci et al. 2001), 21% in Central Asian populations (205 individuals) (Comas et al. 1998) and is less than 1% in a Mongolian population (Kolman et al. 1996).

The HV1 of subject 2 has not been found among the 401 European individuals and the 205 individuals from Central Asian populations. Nevertheless, sequences which have mutations in particular at bases 16223, 16278 and 16362, represented 6% in Central Asian populations, 3% in a Mongolian population (Kolman et al. 1996) and have not been found in the European population tested. Haplogroup X is characterised by 16223T and 16278T, but the 16223T-16362C motive could also be the Asian haplogroup D (Forster et al. 1996). Haplogroup X represents around 4% of European mtDNA (Torroni et al. 1996) and much higher percentages in north Amerind mtDNA (Brown et al. 1998), and has recently been discovered in Asians (Altaïns from South Siberia) (Derenko et al. 2001). Haplogroup D is the most frequent in Asian populations (Starikovskaya et al. 1998; Comas et al. 1998). Further typing of HV2 or RFLPs must therefore be performed to decide on the haplogroup and on the geographic context of the mtDNA motive of subject 2.

Following these results on mtDNA, the origin of the man (subject 1) cannot be established although the reference sequence is the most frequent in Europe. Nevertheless, the woman (subject 2) may have an Asian origin, since 16223T and 16362C are the most frequent mutations in eastern Asian population with around 52% and 35%, respectively (Comas et al. 1998).

Comas et al. (1998) studied mtDNA of populations living along the ancient Silk Road and noted that mtDNA sequences present intermediate features between European and Eastern Asian sequences, probably due to mixings during or before the Silk Road trade. These observations could explain the sequence of subject 2. The hypothesis of mixings between Scythians and distant countries is supported by the discovery of several objects with a Chinese inspiration in the Saka grave.

Conclusion

This study showed that it is possible to amplify DNA from bone samples more than 2000 years old, discovered in a frozen grave. During all the steps of this experiment from the excavation to the run on automatic sequencer, all precautions and controls against contamination were taken in order to obtain authentic results. The sex determination by amelogenin confirmed the morphological sex determination, in agreement with our results being genuine rather than derived from modern contamination. We managed to demonstrate by genetic analysis of several nuclear STR and mtDNA that the two skeletons were not close relatives, but they could be a man and his wife. It is important to note that the analysis of the 13 different STRs and the mitochondrial sequence was necessary to highlight the relationship in this double inhumation. The limited use of commercial amplification kits, very useful for identification test in criminal cases, was insufficient for ancient DNA studies, notably due to uncertainties, when archaeological and kinship contexts are lacking. In archaeological cases, to have a chance to better understand ancient behaviour, it appears necessary to compare and add results from both nuclear DNA and mitochondrial DNA.

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