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Reconstruction of early Neolithic/Bronze Age population diversity in the Shamanka II cemetery at Lake Baikal using mtDNA polymorphism

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Mitochondrial deoxyribonucleic acid (mtDNA) polymorphisms were examined in bone samples of individuals buried in an early Neolithic (c. 5800–4900 BCE) hunter-gatherer cemetery, Shamanka II, located at the southwestern tip of Lake Baikal, Siberia. The main objective was to compare the mtDNA polymorphisms observed at Shamanka II to those previously reported from the Lokomotiv (early Neolithic) and Ust'-Ida (Bronze Age) cemeteries in the same general region also known as the Cis-Baikal. All three cemetery populations comprised remains containing mtDNA haplogroups characterized as being of Asian origin (haplogroups A, C, D, F, G2a, and U5a). The results confirmed that haplogroup frequency distributions among Neolithic populations in southern Siberia were similar, and differed significantly from haplogroup frequency distributions among the Bronze Age populations. Vol. 1, No. 2, 29-35.

KEY WORDS**:** mitochondrial genome, Siberia, Neolithic, Bronze Age

Introduction

Study of ancient DNA (aDNA) has "the allure of time travel" (Pääbo et al., 2004). An investigation in which authentic aDNA is successfully retrieved from human bone samples allows researchers to analyze aspects of the biological history of prehistoric populations. In the past decade, numerous research efforts have concentrated on aDNA methods (Pääbo, 1989, 1993; Rogan and Salvo, 1990; DeSalle, 1994; Austin et al., 1997; Cooper and Wayne, 1998; Kelman and Kelman, 1999; Mooder, 2004; Mooder et al., 2006). With complementary evidence, such as archaeological data, the potential to reconstruct past cultural practices is enhanced by the application of aDNA analysis.

Investigation of aDNA from Siberia is at an early stage. In recent years, there have been several successful attempts at retrieving aDNA from archaeological materials excavated in this region (Uinuk-Ool et al., 2003; Ricaut et al., 2005, 2006; Mooder et al., 2006; Kuch et al., 2007; Snodgrass et al., 2007).

The current study, based on the 2002 field season, examined mtDNA polymorphism among individuals buried in a Neolithic (c. 5000–4000 BCE) hunter-gatherer cemetery, Shamanka II, located at the southwestern tip of Lake Baikal, Siberia (Fig. 1). The major objective of this study was to compare mtDNA polymorphism in material obtained from Shamanka II to the mtDNA results of a study that included sites at Lokomotiv and Ust'-Ida (Mooder, 2004). Lokomotiv belongs to the same cultural group (Kitoi) as Shamanka II, and is contemporary with it (5000–4000 BCE), while Ust'-Ida is representative of the Bronze Age culture (Serovo-Glazkovo, 1000 BCE). There is a pronounced hiatus in radiocarbon dates between the Neolithic and Bronze Age (4900–4200 BCE) cultures in the Lake Baikal region (Weber, 2002). Mooder's (2004, 2006) results revealed a disparate mtDNA distribution between pre- and post-hiatus groups. Because Shamanka II and Lokomotiv are both pre-hiatus Neolithic cemeteries, it is hypothesized that mtDNA results retrieved

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Table 1. Primers used for mtDNA amplification.

Primer	Sequence	Annealing temperature
H16346	5'-CCCATGCTTACAAGCAAGTA	53ºC
L16211	5'-CAGTTTAGGGAAGAGCAGGG	53ºC

from Shamanka II would be similar to those recovered from Lokomotiv and contrast with those from post-hiatus Ust'-Ida.

Material and methods

Shamanka II is the only large Kitoi cemetery to be uncovered outside the Angara valley, and is separated from Lokomotiv by a distance of ca. 80 km. Preliminary non-calibrated radiocarbon dates from Shamanka II skeletal samples suggest that the cemetery was used from approximately 5020 to 4040 BCE (Weber, 2002). The analysis comprised preserved human skeletal remains excavated from Shamanka II during the 2002 field season by Irkutsk State University (Bazaliiskii, 2003).Thirty-nine samples of thoracic and cervical vertebrae, from 29 individuals, were processed.

The likelihood of authentic nuclear DNA being recovered from human bone samples diminishes with time. It is for this reason that mtDNA was the focus of this research, as is the case in many other aDNA studies. MtDNA is far more abundant than nuclear DNA within a single human cell. It is maternally inherited, undergoes little if any recombination, and has a relatively rapid substitution rate (Wallace, 1999). These features make it possible to trace regionally specific maternal lineages, referred to as haplogroups, through time and space.

Specimen preparation

Surface contamination was removed by scraping the exposed outer layers with a sterile scalpel blade, and the samples were washed in 30% sodium hypochlorite three times, with the duration of each time depending on the density of the bone. Subsequently the bone surface was irradiated with ultraviolet (UV) light at 254nm for a minimum of 1h in closed sterile containers. After the surface of the bone sample was cleaned, it was flash-frozen in liquid nitrogen for 20–60 min, depending on sample size. During the freezing process, samples remained in the sealed containers preventing direct contact of the bones with the liquid nitrogen. The sample was crushed with a sterile mortar and pestle to a fine powder. This step is critical in sample preparation, as increased surface area of the material enhances the release of DNA during the extraction process (Boom et al., 1989).

Contamination control

In accordance with the strict precautionary measures outlined by Cooper and Poinar (2000) and Pääbo et al. (2004), all pre- and post-PCR manipulations were carried out in physically separate rooms. The laminar flow cabinet in which the pre-PCR sample preparation was carried out, as well as supplies, pipette containers, and racks, were sterilized prior to each use. Pipette tips and tubes were autoclaved, and reagents exposed to UV light for at least 20 min, to destroy any exogenous DNA. To detect any modern DNA among the samples, both extraction and negative PCR controls were employed. Modern DNA samples (positive control) taken from the investigators were added to the PCR reaction only in the post-PCR room.

DNA extraction

After the sample was crushed, it was subjected to a DNA isolation protocol first outlined by Boom et al. (1989), with modifications suggested by Mooder et al. (2006). Specifically, a silica/guanidiumisothiocyanate (GSN) extraction technique was employed. For each extract, 0.5g of bone powder was incubated in 1000µl of extraction buffer at 65°C for 24h. For silica binding, the bone supernatant was incubated with 500 µl of extraction buffer and 40 µl of

Table 2. Asian mtDNA haplogroup, RFLP variation.

	M positive				M negative			
Haplogroup	C	D	E(G2a)	A	F	B	I(U5a)	X
Enzyme	*HincII*	*AluI*	*HhaI/CfoI*	*HaeIII*	*HincIII*	*N/A*	*HaeIII*	*DdeI*
Positive (+)	uncut	uncut	uncut	cut	uncut	9bp deletion	cut	uncut
Negative (-)	cut	cut	cut	uncut	cut	No deletion	uncut	cut

silica and stirred for 2 h. The resulting silica pellet was washed five times: twice with wash buffer and twice with 70% ethanol followed by a single wash with acetone. After the silica pellet was dried, the DNA was removed with 100 µl distilled UV treated water in a 56ºC water bath for 1 h. The DNA extracts were stored at −20ºC until analysis. For each sample, mtDNA extraction was carried out in duplicate, in order to detect the presence, if any, of random contamination and post-mortem DNA damage.

PCR amplification

The mtDNA extracts were amplified by polymerase chain reaction (PCR). All PCR amplifications were completed on an MJ Thermocycler in 50µl reaction volumes. Each reaction contained 1.25U of 10x PCR Buffer (Invitrogen), 1.5mM of $MgCl_2$ (Invitrogen), 15µg bovine serum albumin (BSA; NEB), 0.2mM of each dNTP (PE Biosystems), 200pmol of each relevant primer, and 1.25U of Platinum *Taq* Polymerase (Invitrogen). The DNA extracts were not quantified. Instead, 8µl of DNA sample template was added to each PCR reaction mixture. The reaction conditions were initial denaturation at 95°C for 2 min followed by 50 cycles of 95°C for 1 min, 54°C for 90 sec, and 72°C for 1 min.

The primers (Table 1) utilized a target 176bp region of the mitochondrial hypervariable region I (HVI) from positions 16191 to 16367. This region of the genome was selected because it contained a majority of the Asian-specific mutations in the mtDNA HVI, which can be detected using a direct DNA sequencing method.

mtDNA haplogroups

Generally, Siberian mtDNA is classified as East Asian due to the region's geographic location. Kivisild et al. (2002) state that East Asian mtDNA belongs to two super haplogroups: M and N. These two are described as the trunks of the analogous phylogenetic tree. The M trunk encompasses the known Asian-specific haplogroups, including C, D, E, G, and Z, while the N trunk includes haplogroup branches A, B, F, and Y.

The methodological framework for the genetic module of the Baikal Archaeology Project (BAP) was developed by Fiona Bamforth and Karen Mooder (Human Identification Laboratory for Archaeology, University of Alberta Hospital, Alberta, Canada).Initially, a combined strategy of RFLP and HVI sequencing of the mtDNA of ancient Siberian samples was utilized. Since HVI sequencing alone was deemed to be more cost- and time-effective as well as an independently reliable means of haplogroup determination for ancient samples, it was later decided that the RFLP approach was unnecessary. However, RFLP classifications were obtained for 4 samples for comparison of results with those of HVI sequencing.

The RFLP process involved the addition of a selected restriction enzyme to the amplified aDNA product. In combination, the mixture was incubated in a water bath at 37ºC for 16 h. Samples were subsequently run on a 10% polyacrylamide gel. The gels were stained with ethidium bromide to visualize results under UV light. The Asian mtDNA-specific restriction enzymes and their features are provided in Table 2.

Of the 29 individuals represented in the samples, 25 consistently amplified in duplicate, and were subsequently sequenced. Of these 25, 21 (84%) produced reliable results, which were associated with Asian-specific haplogroups. Four showed some traces of contamination with investigator DNA and were eliminated from the study. Sequencing of the mtDNA HVI was

Table 3. Some of the reportable HVI sequences found in the Shamanka II cemetery

Haplogroup	16223	16227	16232	16249	16256	16262	16270	16278	16288	16290	16298	16304	16311	16319	16327	Shamanka II
CRS	C	A	C	T	C	C	C	C	T	C	T	T	T	G	C	(n=13)
A	T									T				A		2
C											C				T	1
D	T									T			C	A		4
F			A	C								C	C			3
G2a	T	G				T		T								1
U5a					T		T									1
Other									C							1

conducted using primers (Table 1) bordering a 176bp region of HVI, np 16191–16367. The primary PCR products for cycle sequencing were purified using the Quickstep 2 PCR Purification Kit (Edge Biosystems). Sequencing of 75 ng of template was performed for both the H and L strands, using an ABI 377 sequencer and the Big Dye Terminator v. 3.1 kits (Applied Biosystems). The resulting sequence data were read manually, and differences from the Cambridge reference sequence (Anderson et al., 1981) were calculated (Table 3).

Statistical analysis

In order to test the hypothesis concerning associations of mtDNA polymorphism frequencies with culture group membership, exact tests of population differentiation (Excoffier et al., 2005) were computed using *Arlequin* 3.01 software. The exact test of population differentiation is considered analogous to a Fisher's Exact Test with a 2×2 contingency table; however, the *Arlequin* 3.01 model expands to a table of size defined by the number of haplogroups examined in this study (Mooder, 2004).

Amelogenin analysis

The amelogenin locus was the target of an investigation to resolve the sex of Shamanka II samples with undetermined or ambiguous osteological sex classification. Following the protocol of Mooder (2004), the mtDNA extracts of the ambiguous Shamanka II samples were subjected to PCR amplification consisting of 65 cycles in 50µl reactions. The reaction mixtures were composed of 6µl 10x PCR buffer (Invitrogen), 20µg BSA (NEB), 2.5Mm $MgCl_2$ (Invitrogen), 0.2mM of each dNTP (PE Biosystems), 200pmol of each primer (DNA synthesis laboratory, University of Alberta), and 2U of Platinum Taq polymerase (Invitrogen). The amplified amelogenin products were visualized on polyacrylamide gel (12%) and were given respective molecular sex classifications.

Results

Sequencing results for the HVI region of the mitochondrial genome for the Shamanka II selected samples are shown in Table 4. Of these 21 individuals, the greatest proportion (n=7, or

Fig.1. Location of Neolithic Cis-Baikal cemeteries used in this study.

Table 4. Relative frequencies of mtDNA haplogroups in Bronze Age and Neolithic Populations of southern Siberia.

Haplogroup	N	A	C	D	F	G2a	U5a	Non- Asian
Shamanka II	21	3	2	7	5	1	3	0
		14%	10%	33%	24%	5%	14%	0%
Lokomotiv	31	4	1	7	15	1	1	2
		13%	3%	23%	48%	3%	3%	7%
Summary								
Neolithic	52	7	3	14	20	2	4	2
		13%	6%	27%	38%	4%	8%	4%
Bronze Age	39	10	11	2	3	4	0	9
		26%	28%	5%	8%	10%	0%	23%

33%) belonged to haplogroup D. Five individuals (24%) belonged to haplogroup F. These two haplogroups represented more than half of the individuals analyzed. The mtDNA haplogroup distribution shows a relatively high degree of diversity for such a small sample size. However, the uncalibrated radiocarbon dates suggest a time scale for cemetery use of approximately 1000 years.

As mentioned, RFLP classifications were obtained for four samples. Two of these individuals were classified as haplogroup D, based on a positive cut at 100bp using restriction enzyme *Dde I*, followed by an uncut band at 110bp using the *Alu I* enzyme, followed by a 180bp fragment using enzyme *Hinc II*. One individual was classified as haplogroup A, based on an uncut band at 120bp using enzyme *Dde I* followed by a 76bp fragment after treatment with enzyme *Hae III* (Tables 2 and 4).

The preliminary mtDNA results obtained from Shamanka II did not appear to be spatially organized to overtly reflect matrilineal relationships. Males and females were not equally represented. A four-to-one ratio of male to female burials was found.

Population affinities of Shamanka II and Lokomotiv (Kitoi cultural groups)

The main objective of this study was to compare the mtDNA polymorphism observed at Shamanka II to those from Lokomotiv and Ust'-Ida. Because Shamanka II and Lokomotiv are both prehiatus Neolithic cemeteries, it was hypothesized

that the mtDNA results retrieved from Shamanka II would resemble those recovered from Lokomotiv. The mtDNA polymorphism retrieved from the prehistoric Kitoi remains at Shamanka II mirror the mtDNA results obtained by Mooder (2004, 2006) from the Kitoi burials at Lokomotiv. Haplogroup F was the most common type of mtDNA recovered from Lokomotiv, followed by haplogroup D (Mooder et al., 2006). At Shamanka II, haplogroup D was most common with slightly fewer in haplogroup F. In both cases, haplogroup D and F accounted for more than 50% of the mtDNA polymorphism recovered from the sample population (Table 4). To test the affinity between Shamanka II and Lokomotiv individuals, exact tests of population differentiation (Excoffier et al., 2005) were computed using the *Arlequin* 3.01 software. This approach showed that the mtDNA haplogroup distributions of the Kitoi at Shamanka II and the Kitoi observed at Lokomotiv did not significantly differ (P = 0.9; Table 4).

Population affinities of Shamanka II and Ust'-Ida (Kitoi and Serovo-Glazkovo cultural groups)

The discovery of a gap in the Cis-Baikal mortuary record, initially observed when analyzing radiocarbon dates, inspired the formulation of a biological discontinuity hypothesis (Weber, 2002). Although, as seen in Table 4, the Neolithic Kitoi (Shamanka II and Lokomotiv) share identical haplogroups with the Bronze Age Serovo-Glazkovo, the frequency distributions between pre- and post-hiatus cemetery groups were very different. Using an exact test approach in *Arlequin*, the mtDNA haplogroup distributions of Kitoi versus Serovo-Glazkovo were found to differ significantly (P=0.000). Thus, the general model for biological discontinuity between the pre- and post-hiatus Cis-Baikal populations is not disputed.

Discussion

This study reports the successful retrieval of authentic mtDNA polymorphism data from 21 individuals whose non-calibrated radiocarbon dates range from 5020 to 4040 BCE. The principal objective of this research was to compare the mtDNA polymorphism observed at the Shamanka II cemetery to mtDNA results from the Lokomotiv and Ust'-Ida cemeteries. All three cemetery populations comprised mtDNA haplogroups of Asian origin, but the frequency distribution of each haplogroup differed significantly between the pre-hiatus Kitoi and the post-hiatus Serovo-Glazkovo cultural groups.

The amelogenin locus was the target of an investigation to resolve the sex of Shamanka II samples in cases of undetermined or ambiguous osteological sex classifications. Eleven samples were subjected to amelogenin analysis; nine of the eleven amplified consistently, and had reproducible results over three independent trials. Though evidence for sex inequity has been proposed for Kitoi groups, the females in both single and double graves at Shamanka II possessed accompanying grave inclusions, ranging in number from 14 (grave 11) to 146 (grave 14). These included items such as lithic blanks and antler harpoons, revealing that such items were not exclusive to male graves. Additionally, all females sampled at Shamanka II were determined to belong to different mtDNA haplogroups. Although these two features are intriguing and invite further investigation (e.g., Y chromosome and STR markers), exogamy in marriage practices is implicated through these early results from Shamanka II.

Overall, analysis of both matrilineal and patrilineal DNA markers is still necessary in order to characterize post-marital associations. With more information, the intent would be to explore whether it is possible to determine marriage practices and post-marital residence patterns for both the pre- and post-hiatus groups.

Subtle differences in mortuary customs were observed between the Neolithic Lokomotiv and Shamanka II cemeteries. This variance serves to emphasize the social complexity of prehistoric hunter-gatherer groups, in that while they may be classified as the same cultural group, based on mortuary protocol and biological/genetic affinity, socio-cultural customs, especially those that govern mortuary ritual, are not static overtime and space. These unique and defining features of Lake Baikal region cemeteries add to the intrigue of funeral sites in general, and warrant continued research.

In conclusion, the results of this project have produced a small snapshot of a much larger picture that is currently incomplete. Further excavation and analysis of Shamanka II materials are ongoing, serving to enhance understanding of mortuary customs and biological relationships among the Neolithic Kitoi in this region.

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