PLISSN 0081-3834

SPRAWOZDANIA ARCHEOLOGICZNE

INSTYTUT ARCHEOLOGII I ETNOLOGII POLSKIEJ AKADEMII NAUK

KRAKÓW 2020

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KRAKÓW 2020

Editor's Address
CENTRE FOR MOUNTAINS AND UPLANDS ARCHAEOLOGY, INSTITUTE OF ARCHAEOLOGY AND ETHNOLOGY, POLISH ACADEMY OF SCIENCES, 31-016 KRAKÓW, UL. SŁAWKOWSKA 17

Ministry of Science and Higher Education
Republic of Poland

Volume financed by the Ministry of Science and Higher Education from the founds for science popularization activities, Contract Nr 651/P-DUN/2019

PL ISSN 0081-3834
DOI: 10.23858/SA/72.2020.2

Printed by
PARTNER POLIGRAFIA Andrzej Kardasz, Grabówka, ul. Szosa Baranowicka 77, 15-523 Białystok

Edition: 300 pcs.

Sprawozdania Archeologiczne is regulary listed in the: SCOPUS, CEJSH, ERIH Plus

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Sprawozdania Archeologiczne is regulary listed in the International Current Awareness Service: Anthropology. Selected material is indexed in the International Bibliography of social and Cultural Anthropology.
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IBZ – International Bibliography of Periodical Literature
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Dedicated to Professor Jan Machnik for His 90th Birthday

SPRAWOZDANIA ARCHEOLOGICZNE 72/2, 2020
PL ISSN 0081-3834
DOI: 10.23858/SA/72.2020.2.1844

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"RAPTUS SABINAE?" COMPLEMENTED: MOLECULAR GENETIC STUDIES ON A FEMALE *CALVARIUM* OF THE *BANDKERAMIK* SETTLEMENT OF ROVANTSI IN VOLHYNIA (UA)

ABSTRACT

Mazanec J., Hummel S., Saile T. 2020. "Raptus Sabinae?" complemented: molecular genetic studies on a female *calvarium* of the *Bandkeramik* settlement of Rovantsi in Volhynia (UA). *Sprawozdania Archeologiczne* 72/2, 201-211.

A fragmented human cranial calotte was discovered in a Bandkeramik (LBK) settlement context at Rovantsi in Volhynia (UA). The female *calvarium* of a mature woman with an age of about 45-50 years was uncovered in the deepest part of a settlement pit. It can be dated to round about 5,250 BC. PCR-based molecular genetic analyses were successfully performed on these extremely rare skeletal remains from the Early Neolithic of Ukraine. The female family line can be assigned to haplogroup T2, in which it represents the lineage T2c1d+152. The woman was lactose intolerant, like most LBK individuals. Her hair colour was brown, and her eye colour was found to be hazel.

Keywords: ancient DNA (aDNA), human mitochondrial DNA haplogroup, DNA profiling, polymerase chain reaction (PCR), Early Neolithic, Bandkeramik (LBK), Ukraine

Received: 12.04.2020; Revised: 22.05.2020; Accepted: 28.06.2020

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INTRODUCTION

In a previous volume of this journal, the female *calvarium* of Rovantsi was presented (Bardetskiy *et al.* 2017). It belongs to a mature woman with an age range of *ca.* 45-50 years. The missing jawbone and face of the *calvarium*, as well as some cut-marks, indicate that it had already undergone several post-mortem processes before it found its way into a settlement pit (Fig. 1). The fragmented skull was located about 2.6 m below ground level, in the deepest part of pit 19 north of house I, which had been dug almost 3 m into the loess (Bardetskiy *et al.* 2017, 239, fig. 4). Due to the calcareous soil conditions, the preservation of the bone can be characterized as very good.

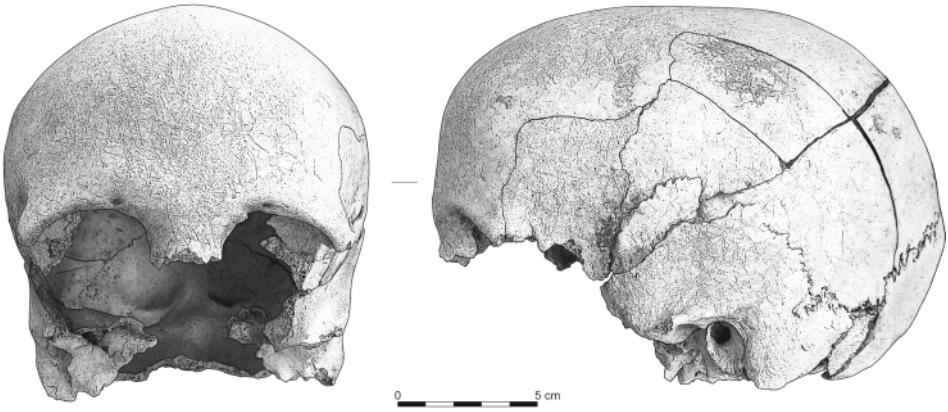

Fig. 1. Rovantsi (UA). Female *calvarium* from pit 19

The site, which lies on a flat loess terrace upon the right bank of the Styr River, is rich in remarkable finds and can be dated to a later stage of the Želiezovce phase, *i.e.* to the 52nd-51st century BC. The skull fragment was found in pit 19 together with Šarka type pottery (Bardetskiy *et al.* 2017, 237, fig. 5; Saile *et al.* 2018, 30-31, fig. 4-5). Two radiocarbon dates obtained from samples of the *calvarium* placed its origin in the 53rd century BC (KIA-53054: 6287±29 BP; MAMS-35954: 6263±29 BP). At first sight, the dates seem to be too old. However, the discrepancy between the death of the woman and the time of the pit's formation may be explained by a long-term ritual use of the body after the death of the person. About five generations later, the human remains were finally deposited in the settlement pit mentioned above. Taphonomic processes explain the difference between the archaeological ceramic dating and the dating of the bone determined by natural science.

Until today the skull fragment is the easternmost preserved human skeletal remain with a *Bandkeramik* cultural affiliation (Dębiec and Saile 2015).The dimensions of the robust skull differ from the expected values of the more gracile LBK average (Bardetskiy

et al. 2017, 246-250). May this be understood as a reference to a Mesolithic background of the person? Since immigrant LBK settlers introduced agriculture, cattle breeding and a sedentary way of life not only in Central Europe but also in the landscapes northeast of the Carpathians, the question of their relationship to the Mesolithic population may claim special interest.

So far, analyses of ancient DNA from numerous human skeletal finds of the LBK have resulted in the identification of genetic features of the Early Neolithic. These characteristics range from phenotypic to metabolic traits as well as maternal and paternal haplotype distributions, which among other things confirms the hypothesis of immigration from the Near East to Central Europe (Haak *et al.* 2010; Brandt *et al.* 2013; Mathieson *et al.* 2015). The very good bone preservation of the Rovantsi find made successful DNA analyses appear possible. Therefore, genetic analyses based on polymerase chain reaction (PCR) and a genome-wide assessment of single nucleotide polymorphisms (SNPs) were performed. We believe that the results obtained should be of interest to those studying the Early Neolithic of East Central Europe.

SAMPLE PREPARATION, DNA EXTRACTION AND ANALYSES

The genetic analyses were performed on the left petrous bone of the *calvarium*. For this purpose, a small piece weighing less than one gram was cut out from the cranial rim of the *pars petrosa ossis temporalis*.

To remove any possibly adhering modern DNA, the sample was immersed in a 6% NaClO solution (sodium hypochlorite) for 5 min., then rinsed in bi-distilled water for 15 min. and dried overnight at room temperature. The sample was then ground to a fine powder for 20 s at 24 Hz. in a ball triturator (MM200, Retsch®). The following preparation and all steps of the analysis were carried out repeatedly to ensure the reproducibility of the results.

Four aliquots of 0.25 g each of the powder were incubated in 3900 µl EDTA (0.5 M, pH 8.3) including 100 µl of Proteinase K for 18 h in order to chemically disolve the inorganic apatite component of the bone powder and to break down the protein backbone of the bone cells and their nuclei. The subsequent steps of DNA isolation and purification were conducted following a standard protocol (Frischalowski *et al.* 2015; Flux *et al.* 2017). This protocol starts with an organic extraction using phenol and chloroform and is continued in the QIAvac extraction system (Qiagen®) using MinElute® columns in order to separate the DNA molecules and to reduce the finale volume of the extract to 50 µl.

A PCR approach was chosen for the analysis of the regional origin of the maternal lineage, the investigation of the lactase persistence, and the proof of authenticity of the ancient DNA through short tandem repeat (STR) typing (Hummel 2003; Butler 2015). All PCRs were done from two to four DNA extracts. The reagent mixtures and amplification proce-

dures followed the standard protocols for the amplification of ancient DNA, i.e. the primers were designed with particular respect to enhanced sensitivity and specificity and up to 40 amplification cycles were performed (Hummel 2003).The analyses included the processing of an extraction blank (EB), no template controls (NTC), and positive controls (PC).

A genome-wide single nucleotide polymorphism (SNP) typing was used to detect the pigmentation of hair and eyes. For this, a set of 24 SNPs (*e.g*. Kayser 2015; Söchtig *et al.* 2015) located on different human chromosomes was analysed using Fluidigm® technology (Schmidt *et al.*2020).

ANCIENT DNA AUTHENTICATION

The STR typing, also known as genetic fingerprinting, was performed using a mini STR heptaplex kit (Seidenberg *et al.* 2012), including six highly polymorphic markers and the amelogenin gene indicating biological sex (Table 1). Due to the unique combination of alleles in the bi-allelic markers, proof of authenticity of the ancient DNA was also achieved.

Moreover, the results of STR typing already indicated that the DNA showed the characteristic signs of degradation. In the agarose gel electrophoresis of the STR amplification products (Fig. 2), this can be deduced from the comparatively weak signal strengths, although the allele determination of all four analytic approaches led to a complete STR typing (= "consensus" conf. Table 1). However, a closer look at the individual results also reveals typical signs of DNA degradation. While some of the single STRs (*e.g*. D21S11 and D5S818) occasionally show allelic dropouts, in FGA (2nd PCR conf. Table 1) even an allelic drop-in can be observed (Butler 2015). Both types of events were attributed to the presence of only small amounts of intact indigenous DNA, which is a function of the age of the skull.

Table 1. Heptaplex typing for authentication

Amplification of Amelogenin and STRs	Extract no.	Sex determination	STR markers					
		Amelogenin	D13S317	D21S11	D18S51	TH01	D5S818	FGA
1.PCR (a)	1	X/-	11/-	31.2/32.2	12/-	6/9	11/-	19/-
1.PCR (b)	1	X/-	11/-	31.2/-	12/-	6/9	11/12	19/-
2.PCR	2	X/-	11/-	32.2/-	12/-	6/9	11/-	21/-
3.PCR	3	X/-	11/-	31.2/32.2	12/-	6/9	11/12	19/23
4.PCR	4	X/-	11/-	31.2/-	12/-	6/9	11/12	19/23
Consensus		X/X	11/11	31.2/32.2	12/12	6/9	11/12	19/23

Key: in some of the genetic markers (Amelogenin, D13S317, D18S51) only one allele occurred in the multiple amplifications, hence, the marker was considered to reveal a homozygous genotype; in other markers (D21S11, D5S818), which were considered to reveal a heterozygous genotype, occasionally allelic dropouts of one of the alleles occurred in single amplifications due to DNA degradation

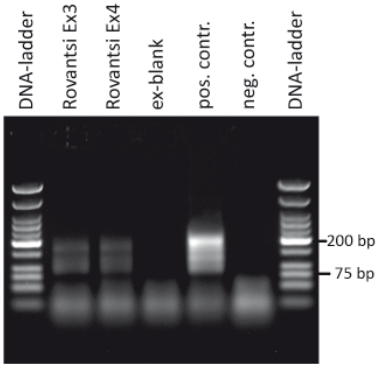

Fig. 2. Electrophoresis of the Heptaplex amplification products of PCRs 3 and 4. After 35 amplification cycles, comparatively faint bands are visible for the Rovantsi sample on the gel. Additionally, control samples were carried along in the analysis

MATERNAL FAMILY LINEAGE

Two fragments of mitochondrial hypervariable regions (HVRs) were analysed to determine the maternal lineage. HVR I was studied by amplification and sequencing of a 435-base-pair (bp) fragment from extracts 1 and 2, while HVR II is represented by a 379 bp fragment amplified from all four extracts. For the Taq cycle sequencing, readily prepared kits (Big Dye® Terminator kit v1.1, Applied Biosystems®) and standard procedures were used. For both fragments, the respective amplification primers served as forward and reverse sequencing primers (*e.g*. Fehren-Schmitz *et al*. 2010; Seidenberg 2016).

The amplification and sequencing analyses were fully reproducible and enabled clear basecalls, although signs for DNA degradation (Hofreiter *et al*. 2001) are visible in the sequences of the HVRs (Fig. 3). Characteristic is the so-called deamination, whereby cyto-

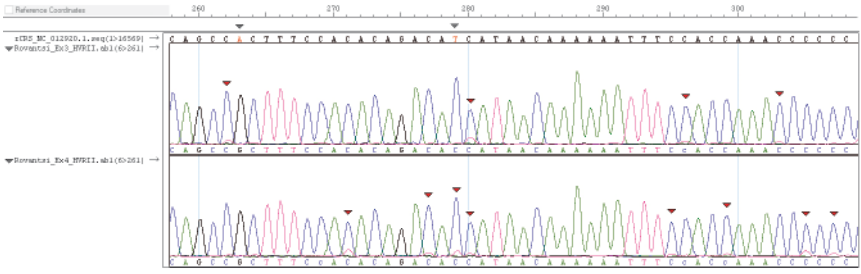

Fig. 3. Part of the HVR II sequence. Nucleotide positions with signs of weak deamination are marked by red arrows. Characteristic are small red peaks (Thymine), which can be recognized underneath some of the large blue peaks (Cytosine). Further, two deviations from the reference sequence (rCRS) are marked with grey arrows. The deviations in the Rovantsi sample sequence belong to the polymorphisms characteristic for the Haplogroup T2c1d+152

Table 2. Mitochondrial sequence and haplotyping

Amplifications of HVRs I and II	Extract no.	Nucleotide positions in HVRs I and II											Haplotype
		T 16126	C 16292	C 16294	A 73	G 143	T 146	T 152	A 263	T 279	del 309.1	del 315.1	
1.PCR HVR I	1	C	T	T									
2.PCR HVR I	2	C	T	T									
1.PCR HVR II	1				G	A	C	C	G	C	C	C	
2.PCR HVR II	2				G	A	C	C	G	C	C	C	
3.PCR HVR II	3				G	A	C	C	G	C	C	C	
4.PCR HVR II	4				G	A	C	C	G	C	C	C	
Consensus		C	T	T	G	A	C	C	G	C	C	C	T2c1d+152

Key: Nucleotide positions are given with the bases realized in the human mitochondrial reference sequence (rCRS); del = deletion; the haplotype was determined using the database EMPOP (Parson and Dür 2007)

Table 3. Genotypes and phenotypes for hair and eye colour

SNP Accession No.	rs1129038	rs11538871	rs12203592	rs12821256	rs12896399	rs12913832	rs12931267	rs1393350	rs1545397	rs16891982	rs1800407	rs1805005	rs1805006	rs1805009	rs2228479	rs2378249	rs2402130	rs28777	rs35264875	rs4778138	rs4959270	rs6119471	rs7495174	hair colour	eye colour
Rovantsi	AG	G	C	T	T	GA	C	G	A	GC	G	G	C	G	G	A	A	A	TA	GA	AC	C	A	brown	hazel-brown

Key: SNP Accession No. = SNPs are identified through their accession numbers in the NCBI database GeneBank; hair colour = was identified through the software "Snipper" (Philipps et al. 2007); eye colour = was identified through the use of the softwares "Snipper" (Philipps *et al.* 2007) and "HIrisplex" (Chaitanya *et al.* 2018); hazel = intermediate eye colour ranging from green-brown

sine nucleotides in some of the target sequences of the DNA extract are damaged and therefore misread during amplification, which in turn leads to their replacement by thymine nucleotides.

The results of the sequencing (Table 2) allow us to assign the maternal lineage to the haplogroup T2, and within this group they represent the lineage T2c1d+152.

DIGESTION OF UNFERMENTED MILK

The study of so-called lactase persistence, which genetically determines the ability to digest milk in adults, was carried out by amplifying a 146 bp fragment on chromosome 2, which frames the LCT-13910 locus associated with lactase persistence (Enattah *et al.* 2002). The respective amplification primers are designed for being introduced to an STR multiplex amplification kit, i.e. they reveal a dye labelling in one primer for fragment length analysis (Fulge 2005; Seebode 2010). The co-amplification of STRs along with the Lac-primers also ensure the proof of authenticity for the amplification of this single bi-allelic marker. The Lac-primers replace the amelogenin primers and are designed to detect a mismatch in the 3' region of the upper primer and introduce a cleavage site for enzymatic analysis. The genotyping of the C/T-polymorphism at the LCT-13910 locus was done through an enzymatic digestion and possible cleavage by the endonuclease Hinf I. The function of the cleavage reaction is controlled through the co-digestion of one of the STRs, which is independent from the LCT-13910 locus.

The results of the analysis show that the female individual has a homozygous expression of the C allele (Fig. 4). This indicates that she was lactose intolerant, meaning that she could not digest unfermented milk or milk products.

EYE AND HAIR COLOUR

The SNP typing for determining hair and eye colour was carried out in a so-called high-throughput approach targeting the relevant 24 SNPs. Basically, the standard procedures as provided by the manufacturer Fluidigm® were used. This comprises a multi-step analysis, including a software-based evaluation of the SNP typing. However, the initial enrichment step had been modified by increasing the number of amplification cycles from 14 to 20 to enable the investigation of minute amounts of ancient DNA (Schmidt *et al.* 2020).

The analyses showed that the colour of the woman's eyes was hazel, with the hazel usually shifting from light brown to green. In addition, the analysis of the SNPs that determine hair colour revealed a brown appearance (Table 3). Both phenotypic characteristics are consistent with each other (Söchtig *et al.* 2015).

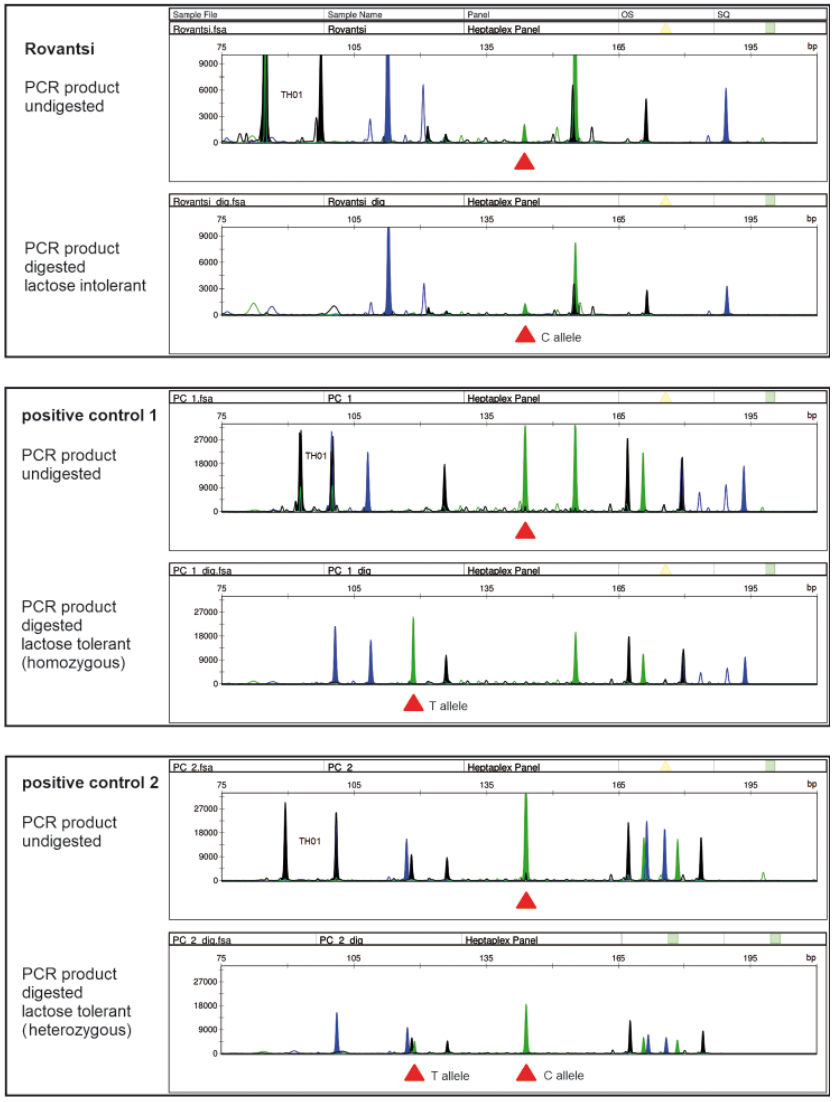

Fig. 4. Electropherograms of the Rovantsi sample and two positive controls showing the results of the lactose persistence investigation. The relevant peaks are marked by red arrows (121 and 146 bp); all other peaks represent co-amplified STRs, including the TH01 locus. Lactose intolerance is indicated by the unaffected peak at 146 bp after enzymatic digestion, representing the C allele. Lactose persistence is indicated by either a fully digested PCR product (PC1, homozygous, T allele) or a partially digested PCR product (PC2, heterozygous, C and T alleles). The homozygous state of lactose persistence can be recognized through the complete disappearance of the peak from its original position at 146 bp and the appearance of a peak at 121 bp. In the heterozygous case only part of the PCR product is digested, leading to a diminished peak height at the 146 bp position (C allele) and the additional occurrence of a peak at the 121 bp position (T allele). The success of the enzymatic digestion is monitored by TH01, which is co-digested in the process and thus disappears from the electropherogram

RESULTS AND DISCUSSION

Mitochondrial DNA haplotyping revealed the identification of lineage T2c1d+152, a subgroup of the haplogroup T2. This has a Near Eastern origin and is thought to have spread to Europe about 10,000 years ago (Pala *et al.* 2012; Strien 2018, 18). It is one of the most frequent haplogroups found in LBK contexts (Haak *et al.* 2005; Haak *et al.* 2010; Brandt *et al.* 2013). The investigations also showed that the individual was lactose intolerant, which, in contrast to today, was the common genetic disposition of European sat the time (Mathieson *et al.* 2015). The analysis of 24 SNPs made it possible to determine the hair colour of the woman as brown and her eye colour as hazel. Both phenotypic characteristics are mutually consistent (Söchtig *et al.* 2015). They are in accordance with a Near Eastern origin. These results are similar to those obtained from the majority of the aDNA data analysed so far from this time (*e.g.* Cerqueira *et al.* 2012; Deng and Xu 2018), although the phenotypic characteristics in Early Neolithic Europe appear to have shown considerable variability (Olalde *et al.* 2014). The findings support an assignment of the mature woman to the *Bandkeramik*. The robustness of the skull thus reflects the considerable morphological variability of the individuals of this culture.

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Strien H.-C. 2018.*Westexpansion und Regionalisierung der Ältesten Bandkeramik* (= *Kommunikation und Wandel. Monographien zu Entwicklungen in der Urgeschichte* 1). Kerpen-Loogh: Welt und Erde Verlag.