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# Complex Analysis of 700-Year-Old Skeletal Remains found in an Unusual Grave – Case Report

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#### Abstract

**Aim:** The present study was designed to analyze the 700-year-old human remains from an unusual grave using a combined approach that consisted of anthropological, archaeogenetic, genealogical, mass spectrometry, 3-dimensional (3D) modeling and facial reconstruction methods to confirm or reject several hypotheses about the skeletal remains.

**Methods:** DNA was extracted from the skeleton and amplified using autosomal and Y-chromosome human identification short tandem repeat (STR) kits that were designed for forensic use, and sequence data were obtained from hyper variable region I (HVRI) mtDNA sequencing. Elemental mapping and quantification of investigated elements were performed using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). The Computed Tomography (CT) images of the skull were created in a transversal plane, and the scans were used to create 3D geometric models of the skull. A plastic physical model (a cast) of the skull was produced by rapid prototyping technology, and the model was used for sculptural facial approximation of the studied individual.

**Results:** The Y-chromosome haplogroup of the sample was determined to be E1b1b, and the assigned mtDNA haplogroup was H. LA-ICP-MS and geochemical analysis revealed that the individual consumed plants and meats, except pork. Anthropological examination estimated the age of the individual to be between 45-55 years, and we did not find any traces of disablement or physical anomalies. Interestingly, we were able to produce a facial reconstruction according to the skull.

**Conclusion:** Applying a multidisciplinary approach to the examination of the 14th century material enabled us to retrieve new types of information that helped us to interpret the excavated skeletal remains.

Keywords: Mass spectrometry; Genealogical; Physical anomalies; Anthropological

#### Introduction

During a rescue excavation in March 2010, archaeologists discovered an unusual grave near the main square of Hradek nad Nisou, Northern Bohemia, that was dated to the first half of the 14th century. The male skeleton was found in a shallow (20 cm deep) pit under a layer of 14th century ceramics that were deposited in a manner that was typical for outcasts. The grave-pit was situated outside the area of the cemetery (approximately 0.5 m from the original graveyard wall) and was parallel to the graveyard wall. The body was in a stretched ventral decubitus position with the left arm along the body and the right arm slightly abducted at the shoulder joint, bent at the elbow and the hand clenched in a fist (Figure 1). The head was slightly turned to the left and faced west. The anatomical contexts were not dislocated, and the skeleton was mostly complete, but some hand and foot bones were missing, and the ribs and vertebrae were poorly preserved. Severe fragmentation of some bones and the skull was likely caused by the relatively shallow depth of the grave and heavy construction machinery crossing over top of the grave. Four silver coins were unearthed next to the left arm (Grossi Pragenses from the period of reign of John the Blind [1310–1346, the Luxembourg Dynasty]) (Figure 2).

According to available written sources, the excommunicated, the unbaptized, Jews, Protestants, offenders, individuals suspected of witchery, those who committed suicide, killed and murdered persons, still-born children, executioners, knackers, strangers, disabled or mentally handicapped persons and unknown cadavers were excluded from burial in a Christian necropolis [1].

Burial in a nonritual position disallowed the participation of the deceased in the Last Judgment and resurrection. In addition, burial in a nonritual position could also be evidence of anti-vampiric remedies, which were performed to keep potential vampires (often including

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Received: August 04, 2014; Accepted January 27, 2015; Published February 05, 2015

Citation: Vanek D, Brzobohata H, Silerova M, Horak Z, Nyvltova Fisakova, Vasinova Galiova M, et al. (2015) Complex Analysis of 700-Year-Old Skeletal Remains found in an Unusual Grave–Case Report. Anthropol 2: 138. doi: 10.4172/2332-0915.1000138

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Figure 1: The grave pit with the body in a stretched ventral decubitus position.



Figure 2: Excavated Grossi Pragenses from the period of reign of John the Blind (1310-1346, the Luxembourg Dynasty).

the condemned) from returning to the world of the living and causing harm. The face-down position can also symbolize a penance, which was the case for King Pepin the Short who died in 768 and was buried in the Abbey of Saint Denis north of Paris [2].

The first DNA study of aged material was performed on the 140year-old museum specimen of quagga skin by sequencing 229 bps of mitochondrial DNA [3]. The results of the first ancient DNA (aDNA) analysis of human remains appeared in 1985 when S. Paabo described his successful attempt to retrieve and analyze the nuclear Alu repetitive sequence family DNA from a 2,400-year-old Egyptian mummy of a child [4]. The development of PCR [5,6] boosted aDNA studies, but most studies have examined sequences of the mitochondrial genome [7-9] rather than focusing on the more difficult nuclear DNA. Improvements in molecular biological technologies in the last decade have helped overcome some of the limitations that restricted DNA analysis to mitochondrial DNA, which is abundant in mammalian cells. In addition, improvements in forensic genetics (e.g., inhibitor-free DNA extraction, multiplex PCR, short STR amplicons and qPCR) have enabled archaeologists to test nuclear DNA, including Y-chromosome STR typing [10,11].

Currently available techniques for DNA analysis using "forensic" procedures [11] can be combined with the classical anthropological examination, advanced imaging and CT scanning techniques [12] and isotope analysis [13] to improve the quality of information that is retrieved from ancient artifacts. A multidisciplinary approach to examining ancient human remains can help verify the DNA analysis results and interpret the findings more precisely.

All of the available techniques for bone sample DNA analysis [14] consume some portion of the artifact and must therefore be considered destructive. There is an understandable need to perform a near-perfect documentation (i.e., a CT scan) of the bone prior to performing a DNA analysis to preserve information that might be important for the anthropological examination.

Anthropological facial reconstruction/approximation is a method that is used to rebuild lost or unknown features of a person's face over the skull. Facial reconstruction is based on a particular relationship between the morphology of a face and the skull beneath it. Facial reconstruction uses average values of soft tissue depths that are measured at defined points of the face and applies various prediction guidelines to determine the size and shape of the facial features (e.g., eyes, nose and mouth).

The present study describes the interdisciplinary approach that was used to identify 700-year-old skeletal remains and resolve the unusual burial pattern of the individual.

# Materials and Methods

# Burial place and specimen

The burial site of the male skeleton from the first half of the 14th century was discovered during a rescue excavation in March 2010 in Hradek nad Nisou, Czech Republic. The skeletal remains are exhibited in museum Brana Trojzemi, Hradek nad Nisou, Czech Republic since 2012. The skeletal remains are in the inventory of the North Bohemian Museum Liberec under the code Pr.c.: 5/2011, Inv.c.:P20316. No permits were required for the described study.

# Laboratory setup, anti-contamination procedures and bone sample preparation

DNA extraction from a femur sample was performed in a specialized laboratory that was specifically dedicated to the analysis of the ancient samples. Vanek et al. have previously described the laboratory setup, the anti-contamination strategies and the preparation of the bone sample [11].

# Silica-based DNA extraction and DNA quantitation

The DNA extraction procedure was modified [11] from a protocol reported by Davoren et al. [14]. The isolated DNA was quantified with real-time PCR using the 4N6 Quant ALU kit (Biologicals, Ricany, Czech Republic) on a MasterCycler® ep realplex S instrument (Eppendorf, Hamburg, Germany).

# PCR amplification and fragment analysis

The extracted DNA was PCR-amplified using the AmpFℓSTR\* NGM<sup>™</sup> PCR Amplification Kit (NGM) and the AmpFℓSTR<sup>®</sup> Yfiler<sup>®</sup> PCR Amplification Kit (Y-filer) (Applied Biosystems, Foster City, CA, USA) and the laboratory-developed Y-miniplex I and II, which consisted of the DYS388, DYS426, DYS444, DYS446, DYS447, DYS449, DYS459, DYS481 loci plus Y-filer<sup>™</sup> overlapping loci – DYS392 and DYS438 [11]. PCR conditions for the NGM  $^{\scriptscriptstyle \rm M}$  and Y-filer  $^{\scriptscriptstyle \rm M}$  kits (Applied Biosystems, Foster City, CA, USA) were in accordance with the manufacturer's recommendations. The only change was that the cycle number was increased by one for both kits. All of the amplifications were performed

on a MasterCycler® *ep* gradient S thermocycler (Eppendorf, Hamburg, Germany).

# **Capillary electrophoresis**

All of the amplified samples were purified using a MinElute PCR Purification Kit (Qiagen, Hilden, Germany). The STR fragments were separated on an ABI PRISM<sup>®</sup> **310** Genetic Analyzer (Applied Biosystems) under standard conditions, and the raw data were analyzed using GeneMapper<sup>™</sup> ID software, version 3.2 (Applied Biosystems, Foster City, CA, USA).

# Mt DNA analysis

Amplification of the hypervariable region I of the mtDNA was performed in a reaction volume of 50 µl that contained approximately 50 ng of DNA, 2 mM MgCl., 200 µM dNTPs, 0.2 nM of each primer (sense 5'-CTCCACCATTAGCACCCAAA-3' and antisense 5'-AGAGCTCCCGTGAGTGGTTA-3') and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA). The PCR cycling parameters were 2 min at 94°C, 40 cycles of 30 s at 94°C, 40 s at 56°C and 120 s at 72°C and a final extension for 10 min at 72°C. The resulting PCR product was ligated in a pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The chain termination reaction [15] was performed with a cycle sequencing technique [16] according to the manufacturer's protocol. The sequences were determined using an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA), and the resulting mitochondrial sequence was compared with the revised Cambridge Reference Sequence. The mtDNA haplogroup was predicted by comparing the results with the haplogroup-specific mutation motifs.

# Interpretation of the results

Two independent extractions per sample were performed, and the resulting extract was used for DNA quantitation and a PCR set. The method of replicated analyses [17,18] was used to interpret the resulting STR profiles.

# Y-haplogroup prediction and database searches

Y-chromosome haplogroups were predicted using a method described by Athey [19]. The resulting Y-chromosome haplotypes were compared with the Y-chromosome haplotypes that were accessible in public databases (*www.ysearch.org* and *www.yhrd.org*).

# 3D modeling

CT images of the skull were created in a transversal plane using a Philips Brilliance 16 scanner (Philips Healthcare, Amsterdam, The Netherlands). The images were recorded with a resolution of 512x512 pixels (the pixel size was 0.488 mm, and the distance between the individual slices was 0.5 mm). The machine settings corresponded to a general algorithm that would be used to examine the skull of a living patient. The mandible scans were made in the frontal plane using the same scanning parameters that were used for the skull. The bone segmentation was performed from a series of CT scans in the Mimics<sup>®</sup> 13 program (Materialise, Leuven, Belgium) [20]. 3D geometric models of the skull and the mandible were created in the electronic STL format, which created a 3D mesh of triangles on the solid geometric surface [21].

# **Facial reconstruction**

The facial reconstruction method consists of several slightly

different techniques, and the selection of a particular technique depends on the "input data" (e.g., the photography of the skull, the virtual 3D model and the dry skull), the preservation state of the facial skeleton, the biological profile of the studied individual and the preferences of the practitioner or technical facility. In the present study, the manual British combination technique [22] (Figure 3) was used for the facial reconstruction. A rather eclectic approach and guidelines from several authors [22-28] were used for the present facial reconstruction.

As the first step, a detailed morphological examination of the skull/cast/virtual model was performed using the relevant facial reconstruction measurements. The next step was to complete the missing splanchnocranium areas (e.g., the right-side nasal bone, the anterior wall of the right-side maxilla, the right-side zygomatic arch and the left-side mandible caput), and we made the missing areas symmetrical to the preserved bones from the opposite side. The mandible was fixed to the base of the skull with a 2-3-mm space between the preserved teeth, which corresponded to the relaxed position of the lower jaw, and plaster eyeballs were positioned into the orbits [25,27]. Wooden pegs, which were used to simulate the average soft tissue depths (based on data for Europeans [29]), were attached to the skull model at defined anthropometric landmarks. The facial muscles were then modeled onto the skull in plasticine. This "anatomical" phase was followed by a morphology determination of the facial features. The nose was reconstructed according to guidelines published by Rynn et al. [23]. Predicting the mouth morphology is generally difficult and primarily relies on artistic interpretation, even if some information could be obtained from the configuration of the teeth and the alveolar process. In the present case, the position of the mouth line was estimated according to George [28,29], but the lip height could not be calculated [22] due to the strong abrasion of the teeth crowns; thus, the lip height was approximated in accordance with the whole face morphology and age. The lip width was determined following the rule of Stephan and Henneberg [24]. We also modeled the parotid gland, and the muscle structure was covered by a layer of skin and subcutaneous fat. After the surface was smoothed, we added the ears and the brows. Because we had no information about the hair style, the reconstructed face/head was left without hair (Figures 4-6).

# Age estimation

To estimate the age of the preserved material, we used a method for evaluating the internal structure of the femur according to Szilvássy and Kritscher [30] and a method for evaluating the tooth on its crosssection in the medial plane according to Kilián et al. [31] and Pilin



Figure 3: An unfinished facial reconstruction in the phase before the application of the skin layer.



Figure 4: Completed facial reconstruction (half-profile, plasticine).







and Šturmankin [32]. The upper section of the left femur and the right canine of the mandible were used for the evaluation.

# Tooth cross-section in the medial plane

The evaluation process that was used for the teeth in the present study was based on several methods: Gustafson's Method and scoring of attrition, secondary dentin deposition, root dentin transparency, periodontal disease progression, cementum apposition and resorption of the root apex on the medial plane of thin ground sections of a singlerooted tooth. The total points allotted were used in formulas from studies by Kilián et al. [31] and Pilin and Šturmankin [32].

The height of the tooth embedment in the periodontium was marked at the root of the canine. The tooth was then extracted from its bed, cast into the two-component Dentakryl resin (SpofaDental, Jicin, Czech Republic) and cut in the medial plane using an Isomet 1000 saw (Buehler LTD, Lake Bluff, IL, USA). The points allotted for traits according to the Kilián et al. [31] scale were assessed on the crosssection.

#### **Biological anthropology**

The unearthed skeletal remains were analyzed using a wide range of consequential analyses. The bones were cleaned and arranged into higher morphological units, and all of the recent mechanical damages were consolidated. The next step was to apply the standard biological anthropology methods. The long limb bones yielded an estimated living stature [33-35], and the age at death was estimated based on the pubic symphysis morphology, the dental attrition, the degree of thyroid cartilage ossification and the metamorphosis of the auricular and retroauricular surfaces of the ilium [34-36].

The gender was determined by the presence of well-defined, characteristic masculine cranial and pelvic features, and the sexual diagnosis was confirmed using a range of morphometric methods (probabilistic sexual diagnosis) [37,38] and DNA analysis.

#### Season of death

A dental cement microstructure analysis can be used to determine the season during which a person died. Although dental cement accrues over a person's lifetime, the rate at which the cement accrues is not the same throughout the year. Indeed, the rate is more intensive during the vegetation period (April to October) when plenty of food is available, whereas the growth rate is slow during the dormancy period (November to March) when food is not abundant (the dental cement accumulation is similar to the annual growth rings on trees). The formation of winter accretion begins in November and ends in April, and summer accretion begins to form in May. Annual accretion consists of light summer and dark winter accretions, and accretion results from the diverse actions of cementoblasts, which are affected by the relative mineral and organic composition percentage [39-41]. The dental cement microstructure analysis also requires a determination of the thickness of the individual winter and summer increments, and the thicknesses are used to derive the amount of time that passed since the accretion began to form (from May or November) on the mammal in question [39-41].

# Nitrogen (<sup>15</sup>N/<sup>14</sup>N) and carbon (<sup>13</sup>C/<sup>12</sup>C) isotopes

The ratio of  ${}^{15}N/{}^{14}N$  nitrogen isotopes can determine whether an animal or person was food deprived. Meat contains the most isotopes, whereas cereals contain the least amount of isotopes. The plants with the most  ${}^{15}N$  nitrogen are pulses.

Carbon isotopes provide information about dietary composition. During photosynthesis, C4 and C3 plants convert the carbon isotopes ( $^{13}C/^{12}C$ ) into complex sugars at varying rates. In C3 plants, the  $^{13}C$  carbon isotope makes up -22 to -30%, whereas the value is between -9% to -16% in C4 plants. In Czech Republic, C3 plants consist of trees, fruit trees and rice, whereas the C4 plants are all cereal plants and grasses. Determining the ratio of carbon isotopes can define a person or an animal's diet [42-44]. The method of assesing  $^{12}C/^{13}C$  isotope ratio is the same as used for radiocarbon dating [45].

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# Multielemental analysis and mapping by means of Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS)

Multielemental analysis and mapping of the elements of interest were performed using a UP 213 laser ablation system (New Wave Research, Inc., Fremont, CA, USA) connected to a quadruple-based ICP-MS Agilent 7500 CE spectrometer (Agilent Technologies, Santa Clara, CA, USA). The commercial Q-switched Nd:YAG laser ablation device worked at the 5th harmonic frequency (213 nm). Programmable XY-stages were used to move the sample during the laser ablation. A sample was placed in the SuperCell (New Wave, Fremont, CA, USA) and ablated by the laser beam, which focused on the sample surface through a quartz window. The ablated material was transported by helium (carrier gas) to the inductively coupled plasma, and the helium carrier gas was mixed with argon after the laser ablation cell. The total gas flow was 1.6 l/min. Optimization of the LA-ICP-MS conditions (e.g., the gas flow rates, the sampling depth, the electrostatic lenses and the voltages of the MS) was performed with the glass reference material NIST SRM 612, and we took into account the maximum S/N ratio and the minimum oxide formation (the ThO+/Th+ counts ratio was 0.2%, and the U+/Th+ counts ratio was 1.1%).

LA-ICP-MS analysis was performed on the root section of a human tooth from the remains (Figure 7). The hole drilling mode was used to record the elements' signals. During laser ablation, the sample was fixed in the same position for 7 s. After the laser ablation, the decrease of the recorded signal between the individual spots was set to 8 s. The LA-ICP-MS ablation pattern contains ablation craters of approximately 40 µm in diameter that are placed at distances of approximately 80 µm. The maximum signals were chosen to prepare the 2D maps, and the background was subtracted for each spot. Trends for calculating the intensities were visualized by the software Grams (ThermoFisher Scientific, USA). Laser ablation was performed with a laser fluency of 5 J/cm<sup>2</sup> and a frequency of 10 Hz. The isotopes <sup>23</sup>Na, <sup>24</sup>Mg, <sup>55</sup>Mn, <sup>56</sup>Fe, <sup>57</sup>Fe, <sup>63</sup>Cu, <sup>66</sup>Zn, <sup>86</sup>Sr, <sup>88</sup>Sr, <sup>135</sup>Ba, <sup>232</sup>Th and <sup>238</sup>U were measured using an integration time of 0.1 s/isotope, whereas for <sup>43</sup>Ca, <sup>44</sup>Ca and <sup>31</sup>P, an integration time of 0.01 s/isotope was used. Elemental mapping was realized on the sample surface with a size of 2.9×1.3 mm<sup>2</sup>. The present analysis resulted in an ablation pattern with 612 ablation spots, and we obtained a photograph of the sample (Figure 7) with a Nikon SMZ 1500 (Nikon, Tokyo, Japan). The Sr, Zn and Ba contents were calculated using bone meal (NIST 1486) pressed into the pellet.

# Results

# **DNA** analysis

The extraction protocol that was used for the bone sample yielded measurable DNA quantities, and the DNA from the independent extracts was amplified using the NGM<sup>™</sup> kit (Applied Biosystems, Foster



Figure 7: Photograph of the root of a human tooth from the remains investigated by LA-ICP-MS.

City, CA, USA). The resulting electropherograms (EPGs) were free of contamination and artificial peaks, and the peak heights were sufficient for reliable data analysis. None of the tested extracts provided results in all of the possible STR loci (15+AME). The worst performances were detected for the D16S539, D2S1338, D18S51, FGA and D1S1656 loci.

When we combined the data from the Y-filer<sup>™</sup> (Applied Biosystems, Foster City, CA, USA) and the Y-miniplex I and II, we obtained Y-chromosome haplotypes with 14 STRs (Table 1). None of the extracts provided results in all of the possible STR loci (17+8). The Y-chromosome haplogroup E1b1b [46] was successfully predicted [19] for the tested sample.

All of the STR amplicons were purified prior to capillary electrophoresis using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) (Figure 8).

shows an example of a positive post-PCR cleaning effect and indicates the missing peaks in larger amplicons.

A comparison of the resulting haplotype to the YHRD database [47] did not reveal any matches; however, a comparison with the YSEARCH database (*www.ysearch.org*) resulted in several matches to living individuals (Table 1). The Y-chromosome haplotype was stored in the *www.ysearch.org* database under the sample identifier DE8WN.

The mtDNA HVR1 sequence analysis only revealed one difference (i.e., position 16362C) from the revised Cambridge reference sequence [48,49]; thus, we can predict that the individual belongs to mtDNA haplogroup H [50].

The results of the skeleton mtDNA and Y-chromosome typing were compared with 12 male individuals from the Hradek nad Nisou region who were self-identified as denizens. None of the individuals matched the skeleton's mtDNA haplotype. One person matched the skeleton's Y-chromosome haplogroup, but numerous differences in his haplotype exclude any closer paternal relationship. Table 2 summarizes the results of the Y-chromosome and mtDNA typing of the denizens.

#### **3D modeling**

The 3D modeling was used to perform the segmentation of the bone tissue of the skull and the mandible from a series of CT scans, design a 3D geometric model of the skeleton and place the individual skull bone fragments into the correct anatomical position.

During the image data segmentation, the individual models of most of the skull bones were designed. Because the shape of the skull was partially deformed and several bone fragments were separated, it was necessary to reposition the individual fragments into their original anatomical positions. The following bone fragments were repositioned: *os zygomaticum* (right side), *os temporale* (both sides; both were assembled from a number of fragments), *os parietale* (both sides; the left side was assembled from a number of fragments), *os frontale*, *os occipitale*, and the mandible (Figures 9 and 10).

shows the shape of the skull after repositioning the fragments. The plastic physical model was designed using rapid prototyping (RP) technology for fused deposition modeling (FDM). The physical model was made from white plastic acrylonitrile butadiene styrene (ABS) on a 3D Prodigy Plus printer (Stratasys, Eden Prairie, MN USA). Figure 11 shows the resulting physical model of the reconstructed skull.

#### Age and sex estimation

Although the tested human bones were damaged by decomposition

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Sample ID	Sample/individual name	393	19	391	385a	388	389-1	392	389-2	458	H4	456	635	446	481
DE8WN	Skeleton HnN	13	14	10	16	12	12	11	31	15	11	15	23	11	22
3NWQC	Casales	13	14	10	16	12	13	11	31	15	11	16	Х	12	22
4AK2U	Smith	13	13	10	16	12	12	11	30	15	11	16	Х	12	22
9JM9U	Donovan	13	13	10	17	12	13	11	31	15	11	15	Х	11	22
A2URP	Thugut (Toviah)	13	14	10	16	12	13	11	30	15	11	15	Х	12	22
EUAH6	Urasin	13	13	10	16	12	13	11	32	15	10	15	Х	12	22
KFKGM	Gwozdz	13	13	10	16	12	13	11	32	15	11	15	Х	13	22
KRHRX	Bell	13	14	10	17	12	12	11	29	15	11	15	22	12	x
U5QF4	Modal Ht V13 C	13	13	10	17	12	13	11	32	15	11	15	Х	12	22

Nonmatching alleles are in *italics*.

Table 1: Results from the Ysearch database search.

	Y-chromosome haplogroup	mtDNA haplogroup
Person 1	R1a	T2
Person 2	R1a	
Person 3	R1b	U5
Person 4	E1b1b	Н
Person 5	R1b	Н
Person 6	R1b	Н
Person 7	J2b	J
Person 8	R1a	U3
Person 9	R1b	Н
Person 10	R1b	U5
Person 11	R1b	U4
Person 12	G2c	Н







Figure 9: The original skull.



Figure 10: The virtual model before (left) and after (right) repositioning the right orbit and postmortally deformed neurocranium.



Figure 11: The resulting physical model of the reconstructed skull.

processes and some parts were completely missing, they contained evidence about the body construction, height, gender, age at death and

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health status of the buried individual. The present remains belonged to a robust, well-muscled man whose maximum lengths of the long limb bones yielded an estimated living stature of approximately 170 cm [33-35]. The age at death was estimated to be between 40 and 60 years based on the pubic symphysis morphology, the dental attrition, the degree of thyroid cartilage ossification and the metamorphosis of the auricular and retroauricular surface of the ilium [34-36].

The sex was determined by the presence of well-defined, characteristic masculine cranial and pelvic features, and the sexual diagnosis was confirmed using a range of morphometric methods (probabilistic sexual diagnosis) [37,38] and DNA analysis. Skeletal pathologies included traces of numerous intravital tooth losses on both the upper and lower dental arches, a dental fistula of the mandible corps perforating the front wall (associated with the inflammatory process), pronounced alveolar resorption around the roots (periodontal disease) and slight marginal lipping of the right femoral head (Figure 12).



Figure 12: An X-ray of the upper section of the left femur.

Changes of dental tissue	Score
Attrition	2.00
Periodontitis	1.50
Secondary dentin	1.75
Calculus	1.75
Root resorption	1.50
Transparency	2.25

Table 3: Points according of Kilian's scale.



Figure 13: A thin ground section of the right lower canine.



**Figure 14:** A section of the root of the first permanent incisor of the man from Hradeknad Nisou. The last accretion that is evident is the winter (dark) accretion, which indicates that the man died in the spring (April to May).

Laboratory code UGAMS #	I.D. code	Anatomy	δ¹³C	δ¹⁵N	
7352	Hrad-1	Molar	n/a	n/a	
7353	Hrad-2	Femur	-20.33	9.89	

**Table 4:** Results of the analyses of carbon and nitrogen isotopes from a manfrom Hradek nad Nisou. n/a - no collagen suitable for analysis was present.

Element	Content (mg/kg)	SD
Sr	251	16
Zn	116	19
Ва	50	14

 
 Table 5: Sr, Zn and Ba contents were obtained using LA-ICP-MS in the dentin of a root of a tooth from the remains.

Neither vertebrae nor the bones in the right arm, including the carpals, metacarpals and phalanges, showed any degenerative changes.

Although sophisticated methods are available to determine important demographic data (namely age at death), some limitations exist. For example, the degree of senescence transformation varies and is influenced by many factors, such as inherited dispositions, nutrition and mechanical loading. We could only conclude that the man buried behind the graveyard wall probably died during his fifth or sixth decade. Age categories used by biological anthropologists are relatively wide, and histological methods must be used if there is a need to specify the age more accurately.

An X-ray of the femur (Figure 12) suggested that the compact bone had an intermediate thickness, the medullary cavity slightly exceeded the upper margin of the lesser trochanter and the spongiosa trabeculae in the neck were loosened with visible small cavities. We could not evaluate the spongiosa in the greater trochanter due to material damage, although it appears that loosening also occurred at this site. According to Szilvássy and Kritscher [30], these structures correspond to the maturus I category (i.e., 40-50 years).

The age that was calculated according to methods described by Kilián et al. [31] was 48 years, whereas the age that was calculated according to the methods described by Pilin and Šturmankin [32] was 52 years (53 years after accounting for trait significance) (Table 3). The mean of these three determinations is  $51 \pm 3.5$  years.

Based on the X-ray and tooth thin section evaluation (Figure 13), we concluded that the age of the man was most likely 45-55 years.

#### Analysis of the dental cement microstructure

Two sections of roots from the first permanent incisor (incisivus– $\mathrm{I}^{\mathrm{i}})$  were studied.

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![](_page_7_Figure_2.jpeg)

The last accretion on the first upper incisor of this man was the winter accretion. The summer accretion, which begins to form at the beginning of May, had not begun to form on the first upper incisor [39-41]. Therefore, the man died at the turn of April to May (Figure 14).

# Multielemental analysis and mapping

The isotope analyses were performed at the Center for Applied Isotope Studies, University of Georgia. Table 4 shows the results of the analyses of the ratio of carbon and nitrogen isotopes.

# Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)

The Sr, Zn, and Ba contents were calculated using bone meal (NIST 1486) that was pressed into a pellet. Table 5 summarizes the amounts of these elements in the nonaffected dentin.

The distribution maps of the Sr/Ca, Sr/Ba and Sr/Zn ratios (Figure 15) were normalized to the calcium signal to minimize the laser beam energy fluctuation.

# Discussion

The main goal of the present study was to apply different scientific techniques to confirm or reject theories about the unusual burial pattern of the excavated male skeleton, and we primarily investigated the "Vampire" and "Jewish" theories.

Throughout history, death has often been a surprisingly extended process. During this process, the central entity was the soul rather than the body. At the same time, the soul itself could often behave like a body, and vampires inhabited a curious space between the living and the dead [51-61].

The practice of burying the dead face down has occurred across time and societies, and the earliest known case of such a burial (26,000 years ago) was found in the Czech Republic [52]. Frequent and repeated discoveries of skeletal remains with evidence of antivampiric remedies indicate that the belief in vampires was widespread throughout the medieval Czech territory. In excavated cemeteries, some burials exhibit traces of anti-vampiric measures (including burial in a face down position) to keep vampires from returning to the world of the living [53]. In 1966, an unusual concentration of such graves, where fourteen adult corpses were buried, was discovered in the city of Čelákovice, which is located a few kilometers from Prague [54]. Further re-assessment of this unique burial place infirmed both the dating (late 10<sup>th</sup> and early 11<sup>th</sup> centuries) and the suspected vampirism due to the hypothetical existence of a local execution site [55] and similarities to other European sites, such as the Swiss Emmenbrücke [56]. Abnormal burials and irregularities have been observed in a small number of archaeological sites in early medieval cemeteries in Lahovice [57], Prague-Motol [58], Brandýsek [59] and Radomyšl [60]. An increasing number of both testimonies and precautions against potential revenants have been documented (even in modern history), and ethnographic materials bear evidence of abnormal burials up to the first half of the 20<sup>th</sup> century. Mjartan [61] pointed out the collective character of this folk belief regarding burial practices and different preventive measures in particular regions. Preventive measures may have been performed for individuals who differed either physically or mentally from their contemporaries. There are no traces of disablement or anomalies in the skeletal remains of the man from Hradek nad Nisou, and it is unclear whether he was a stranger, a condemned man or someone professing a religion other than Christianity.

# **DNA** analysis

The success rate of DNA extraction from bone samples is strongly influenced by the chemical composition of the soil, the humidity, the pH, the temperature, the presence of microorganisms and the age of the specimen, and choosing the appropriate DNA extraction technique is crucial for most ancient skeletal samples [11]. According to our previous experience with ancient samples, only the middle part of the femur was used for DNA extraction, and the extraction procedure followed a previously described extraction protocol [11] using the newly designed HaR and DeX buffers (Biologicals, Ricany, Czech Republic). The silica-based extraction procedure provided sufficient amounts of both nuclear and mitochondrial DNA, which was mildly contaminated with co-extracted PCR inhibitors. We used Qiagen columns for the post-PCR cleaning procedure, which significantly improved the resulting EPGs. The AmpFℓSTR® NGM™ PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA) has been proven to be suitable for highly degraded and inhibited samples in different studies [11]. The AmpFℓSTR® NGM<sup>™</sup> PCR Amplification Kit (NGM<sup>TM</sup>) (Applied Biosystems, Foster City, CA, USA) for ancient DNA typing was used in the present study to verify the suitability of this new-generation forensic kit for problematic samples, including those with degraded DNA or PCR inhibitors. Validation studies performed with the NGM suggested that it had a superior performance for challenging forensic samples [62]. We used autosomal STR typing to monitor the possible contamination of the extracted ancient DNA with contemporary DNA. The resulting DNA profiles of the amplified ancient extract did not show any signs of contamination. The NGM  $^{\scriptscriptstyle \rm M}$  kit performed well, even in the presence of soil-borne inhibitors detected by the quantitative RT-PCR approach. Amplification problems for the ancient extracts were encountered at the D2S1338, D18S51, FGA and D1S1656 loci. The most probable explanation might be the relatively long amplicons, but D12S391 typing was successful for all of the tested extracts, and the locus outperformed the D1S1656, which had shorter amplicons. The resulting EPG quality can be greatly improved by the post-PCR cleaning, which not only increases RFUs but also removes some dye-blobs that can mask real peaks. We concluded that the NGM™ kit may be a suitable tool not only for authenticating ancient samples and detecting contamination by modern DNA but also for studying familiar relationships between excavated skeletons [11].

Currently available forensic kits for degraded DNA (miniSTRs) do not cover the Y-chromosome STR loci; thus, we used two laboratory-developed Y-chromosome mini-STR systems [11] that allow DNA typing on highly degraded DNA samples [63]. Although none of the samples were successfully typed in all 26 loci, we obtained a Y-chromosome haplotype with 14 STR markers. The obtained

haplotype was suitable for both the haplogroup determination and the database searches. A direct comparison of the results obtained from the skeletal remains to the denizens of Hradek nad Nisou did not reveal any matches that indicated a relationship to the same parental or maternal lineage. The city of Hradek nad Nisou has approximately 7,500 citizens, and the number of tested denizens comprised approximately 1/1,000 of the city population.

The Y-chromosome haplogroup was estimated using a predictor software tool [19]. Because the haplogroup data that were analyzed in the manuscript were translated from the Y-STR haplotype information, there is a possibility that the prediction software resulted in an incorrect haplogroup assignment [64].

# Analysis of the dental cement microstructure and the ratio of carbon and nitrogen isotopes

Based on the analysis of the tooth cement accretion, we may assume that the man died in the spring (April to May). Indeed, the last accretion was dark, and the (light) summer accretion had not begun to form. As indicated in the table, this man ate all forms of cereal except millet. In addition, he ate fruit (e.g., apples and pears) [39-41] and animals that lived on C3 foods (i.e., a greater extent of the man's meat and milk intake came from sheep and goats compared with cattle) [65] (Figure 15). Based on the isotopic ratios of C to N, the man was not nourished by meat or products of omnivores, such as the domestic pig, which has different isotopic ratios of the above-mentioned elements. Because the man had a variety of foods in his diet, we hypothesized that he had a reasonably higher social status. We also hypothesized that the man lived in a typically agricultural landscape (fields with meadows and woodland islands) [66].

# Multielemental analysis and mapping

LA-ICP-MS utilizes a laser beam for sampling, and material is

![](_page_8_Figure_7.jpeg)

![](_page_8_Figure_8.jpeg)

disengaged during the laser pulse-sample surface interaction. An ablation crater is created on the sample's surface, and the size of the crater depends, for instance, on the matrix, the energy and the wavelength of the laser beam (The ablated mass containing atoms and ions and is led to the next ionization source, such as inductively coupled plasma). Ions are then separated in an analyzer according to their m/z ratios. The advantage of the LA-ICP-MS technique is that it requires little or no sample preparation. In addition, LA-ICP-MS analysis allows both qualitative and quantitative analysis [69]. Importantly, elemental mapping is performed with solid samples. LA-ICP-MS is used in several fields, including geology [68], archaeology [69,70] and biology [71].

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The main component of teeth is hydroxyapatite  $Ca_{10}(OH)_2(PO_4)_6$ , and calcium and phosphorous are matrix elements. The distribution of these elements is homogeneous. In the tooth excavated at Hrádek and Nisou, the calcium content rapidly decrease in the surrounding area of the root channel (Figure 15); however, the original calcium content probably differed from the current value because diagenesis likely affected the root. Similar results were observed in the cementum.

Based on the Sr and Zn contents, we hypothesized that the man consumed both plants and meat. The results of LA-ICP-MS confirmed the outcomes of the geochemical data. The Sr/Ca, Sr/Ba and Sr/Zn ratios were used to estimate the mobility and the diet (Figure 15), and the distribution maps of the ratios were normalized to the calcium signal to minimize the laser beam energy fluctuation. The damaged part of the sample surface was not usable for reconstruction. Near the root channel, variation in Sr/Ca ratio can be caused by changes in the diet. In addition, fluctuations in the ratios showed small intensity changes that corresponded to the unaffected part of the tooth.

Figure 16 explains the Comparison data (Great Britain, Denmark and Vedrovice compared with Danish Mesolithic hunters and historical Eskimos) were obtained from several previous studies [42-44,78,79].

# Age estimation

Evaluating the age at which a person died is one of the basic tasks of examining skeletal material. In the remains of unknown persons, it is not possible to determine the time elapsed from birth or the chronological age; however, the so-called biological age may be estimated. We determined the biological age of the present remains by comparing the sample with remains of a known age, which represents a generalization of the variability of an organism's wear at a certain stage of life (age).

Generally, the biological age at the time of death is estimated using methods based on degenerative changes. The most commonly used method includes changes in the vertebral column, changes in the wear of articular surfaces and structural changes (e.g., changes in the spongiosa structure in the heads of long bones, which has been described by Szilvássy and Kritscher [30-32]). The method of changes in the spongiosa structure in the heads of long bones is quite applicable, although Gehring et al. [72] claimed that an age estimation by the method of Szilvássy and Kritscher [30] should only serve as a rough estimation because lower age estimates occur for individuals over 50 years of age. Forensic cases in which we could re-verify the age following the positive identification of unknown skeletal remains show that the method by Szilvássy and Kritscher [30] is applicable.

Age estimates based on indicators acquired from an examination of the teeth (e.g., counting the layers in the cement [73] or evaluating the prominences on the thin sections) are considered to be the best

Analysis	Results	Vampire hypothesis	Jewish hypothesis
Burial pattern	Face down position	Moderate support	No support
Burial pattern	4 silver coins in right fist	Moderate support	Moderate support
Sex	Male	No support	No support
Normal amplification of STR loci	Yes	No support	No support
Predicted Y-chromosome haplogroup	E1b1b	No support	Moderate support
Predicted mtDNA haplogroup	Н	No support	No support
Predicted age	45-55 years	No support	No support
Direct YHRD database match	No	No support	No support
Direct YSEARCH database match	No	No support	No support
Similarity to V13 modal Y-chromosome haplotype	YES	No support	Moderate support
Match of the Y-chromosome/ mtDNA results with denizens	NO	No support	No support
Isotope analysis	No pork meat diet	No support	Moderate support

Table 6: Summary of the results.

methods for age determination. The method of tooth thin sections was first published in 1947 (internationally in 1950) by Gustafson [74,75], and this work later became the basis for other authors. Kilián et al. [31] further developed this method in the Czech Republic. In addition, Pilin and Štumankin [32] included trait significance, which Kilián did not consider. Although evaluation of tooth thin sections has a slightly subjective component, it is simple, well described and relatively quick. Although age can be estimated using multi-rooted teeth [76,77], singlerooted teeth are usually preferred because it is much easier to produce thin sections from single-rooted teeth.

#### 3D modeling

The calvarium and the mandible were scanned using a spiral CT scanner to generate a three-dimensional model of the skull before the destruction of three teeth and the inner surface of the mandibula for chemical and histological analysis. The model can be archived (if the original is destroyed) and/or transferred to physical reality using either the stereolithographic or FDM copies produced with a 3D printer.

#### **Facial reconstruction**

The facial reconstruction was based on a physical "cast" of a virtual model of the skull after replacing the separated and deformed bone fragments. This approach protected the fragile original skull from damage under the weight of plasticine and allowed the original skull (as well as virtual model) to be used as a reference when most of the facial parts of the plastic model were covered with modeling clay. Another advantage of using a plastic skull "replica" for modeling the facial reconstruction is that the original skull can provide information about the features that may have been lost during the CT scans, the design of the 3D geometric model and the prototyping (e.g., the delicate surface structure of muscle insertion points).

The created facial reconstruction can be scanned with a 3D laser or optical scanner to produce a virtual model of the face. Surface details, including fat content age-related changes, skin color, eye color, hairstyle and facial hair, can be added and modified using 3D graphic software. Researchers can also make a cast of the finished facial reconstruction Page 10 of 12

in plaster, plastic or wax and use it as an exhibit that can be touched.

Because of contemporaneous limitations, the facial reconstruction method can never produce a precise portrait of a person. Even if an approximated face bears a particular amount of individuality (there is a uniquely shaped skull under it), it generally only represents a type of face that the person might have had. Nevertheless, facial reconstruction is a useful aid that allows us to look at the face of a man who lived 700 years ago.

# Conclusion

Table 6 summarizes the results of the multidisciplinary analysis of the skeletal remains.

The use of a multidisciplinary approach allowed us to perform the Y-chromosome and mtDNA analysis of the 14<sup>th</sup> century skeletal remains, estimate the age of the individual, reconstruct the skull and prepare a 3D model to perform the quantitative and qualitative mass spectrometry element analyses. Based on the analysis of the tooth cement accretion, the man died in the spring (April to May). The man's diet was mixed and consisted of a large proportion of C3 plants (e.g., fruits and vegetables) and animals that fed on C3 plants (the man consumed more meat and milk from sheep and goats compared with cattle). The ratio of carbon and nitrogen isotopes also showed that this man lived in a typically agricultural landscape with fields, meadows and woodland islands.

The present study concluded that the "vampire" hypothesis can be rejected because we did not find any significant data (nonhuman mtDNA, Y-chromosome haplotype or STR profile) that would strongly support this hypothesis. The "Jewish" hypothesis is moderately supported by both the Y-chromosome and the element analyses. The procedure that was described for examining skeletal remains of unknown individuals can serve as an example of the available scientific approaches that can be applied to other archaeological, anthropological, or forensic studies.

#### Acknowledgment

We would like to acknowledge the technical help of Dr. Jana Veleminska from the 3D laboratory, Department of Anthropology and Human Genetics, Faculty of Science, Charles University, Prague, Czech Republic. In addition, we would like to acknowledge MUDr. Ladislav Endrych from the CT scan, Department of Radiodiagnostics, Liberec hospital, Czech Republic. We would also like to acknowledge the help of the excavation team, which consisted of Vaclav Kropacek, Mgr. Michaela Bradacova Mgr. Jiri Unger, and Anna Kellerova.

#### Funding

The research was primarily financed by the City of Hradek nad Nisou, Liberec Region, Brana Trojmezi, and the North Bohemian Museum Liberec.

The research was also partly supported by the projects "VZPMMK" (00002327201), Czech Science Foundation grant No. 14-36938G, Ministry of Culture of the Czech Republic grant (DKRVO 2014/18, National Museum, 00023272), and by the European Regional Development Fund project "CEITEC" (CZ.1.05/1.1.00/02.0068).

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