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Research Paper

Searching for the mother missed since the Second World War

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ABSTRACT

The aim of the study was to perform the genetic identification of a human cranium from a Second World War gravesite in Slovenia and find out if it belonged to the mother of a woman used as a family reference. Both genetic and anthropological examinations were carried out. The genetic examination was performed on 2 molars and petrous bone. Prior to DNA isolation 0.5 g of tooth and bone powder was decalcified. The DNA was purified in a Biorobot EZ1 (Qiagen) device. The nuclear DNA of the samples was quantified and short tandem repeat (STR) typing performed using two different autosomal and Y-STR kits. Up to 22.4 ng DNA/g of powder was obtained from samples analyzed. We managed to obtain nuclear DNA for successful STR typing from the left second molar and from the petrous bone. Full autosomal genetic profile including amelogenin locus revealed the male origin of the cranium that was further confirmed by the analyses of Y-STRs. The same conclusions were adopted after the anthropological analysis which identified the cranium as that of a very young Caucasoid male. The male origin of the cranium rejected the possibility of motherhood for the compared daughter. For traceability in the event of contamination, we created an elimination database including genetic profiles of the nuclear and Y-STRs of all persons that had been in contact with the analyzed cranium and no match was found.

1. Introduction

The Commission of the Government of the Republic of Slovenia on Concealed Mass Graves in Slovenia has recently registered almost 600 World War II (WWII) hidden mass graves[1] with approximately 100,000 victims of mass killings of military and political opponents. In some of the gravesites the remains of thousands of people have been discovered, some of them contain the remains of only a few victims and there are also a lot of individual graves. The excavations were carried out at only a few of the Slovenian mass graves.[2] Here a case of post-war killing of a mother from Bohinj is presented. Close living relatives were possible to trace since the executed women left a year old daughter that lived without her mother. Forty years ago a skeleton from an individual grave was excavated in Bohinj region and only the cranium was stored in the store of the Small War Museum. The burying site matched to the witness accounts, but the initial anthropomorphological screening could not determine the sex of the remains.

Recently we have been asked for genetic identification of the cranium by comparison with the living daughter. The genetic analysis was conducted parallel to a more detailed anthropological analysis of the cranium.

Genetic analyses are invaluable in identification of skeletal remains of missing persons, disaster victims and victims of mass fatality incidents. Discovery of skeletal remains dating to the period of the WWII are not uncommon on the territory of former Yugoslavia. Examinations of mass graves in Slovenia,[3–7] Croatia[8] and Bosnia and Herzegovina[9] have been reported in forensic literature. DNA - assisted identifications from this era were done using mitochondrial DNA in the past[10,11] and STR typing analyses in more recent research.[6–9] STR techniques were used for human identification even in more recent ancient analyses.[12,13]

In skeletonized human remains bones and teeth are the only accessible source of DNA which can be preserved for a long time. In them binding of DNA to hydroxyapatite provides stability of DNA and its preservation.[14] Old skeletal remains usually contain minute amounts of DNA, potential inhibitors of PCR reactions are present, DNA became compromised due to degradation, and the exceptional risk of contamination limits the success of DNA typing.[15,16] When

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dealing with skull, petrous part of the temporal bone is usually used in ancient DNA analyses.[17] According to Sosa et al.[18] dental DNA can be better preserved in ancient skeletons than bone DNA and have a better chance of successful genetic typing, probably because of the high degree of protection conferred to the DNA by the enamel. From the comparative study of the performance of nuclear DNA typing of Second World War skeletal remains, we discovered that teeth are most suitable for typing, followed by the femur bones and tibiae,[5,19,20] while petrous bones were not analyzed for Slovenian WWII skeletons yet. For performing this study teeth and petrous bone were used for genetic analyses and the results obtained from teeth were confirmed by the results obtained from the petrous bone. The research project was approved by the Medical Ethics Committee of the Republic of Slovenia (0120-118/2015-2; KME 123/06/15) and informed consents were obtained from living relative and persons to be included in elimination database.

2. Materials and methods

2.1. Anthropological analysis

The skull was subjected to a classical anthroposcopic and osteometric anthropological analysis. The analysis aimed to construct the biological profile of the subject (attest ancestry, sex and age) and to identify any nonmetric trait, pathological state and/or sign of antemortem, perimortem and postmortem trauma that could help the identification process and/or attest the manner and cause of death. The anthropological analysis was conducted according to the published Standards for data collection from human skeletal remains and Scientific working group for forensic anthropology guidelines.[21,22]

2.2. DNA analysis

To prevent and detect contamination with modern DNA and to ensure quality standards in the molecular genetic laboratory, published recommendations were considered when performing cleaning and pulverizing of teeth and petrous bone, DNA extraction, quantification and polymerase chain reaction (PCR) amplification.[14,23–27] Contamination monitoring was included in all stages using blank controls and the elimination database containing all persons that had been in contact with the skull was created to check for authenticity of genetic profiles obtained from the skull. We collected buccal swabs on sterile cotton swabs from the persons included in the elimination database and reference sample. The left second molar, the left third molar and the petrous bone were used for genetic investigations. DNA was extracted from powder using full demineralization protocol as described by Zupanič Pajnič.[27] Following the manufacturer's instructions[28] the Biorobot EZ1 (Qiagen, Hilden, Germany) was used to obtain genomic DNA from decalcified bone and tooth precipitate using the trace protocol and from elimination database and reference buccal swab samples using the “tip dance” protocol.

The DNA extracts were subjected to quantification using the Quantifiler Human DNA Quantification Kit (Applied Biosystems, Foster City, CA, USA). The reactions were carried out using the 7500 Real Time PCR System (Applied Biosystems) with the HID Real-Time PCR Analysis Software, version 1.1 (Applied Biosystems) applying the protocol recommended by the manufacturer.[29] Autosomal and Y-chromosomal genetic profiles were determined after the duplicate PCR amplification using two different autosomal and two different Y-chromosomal kits. The Investigator ESSplex Plus kit (Qiagen) and the AmpFlSTR NGM PCR Amplification Kit (Applied Biosystems) were used to analyze a set of 15 highly variable microsatellite loci and amelogenin sex marker. The two kits contain different sets of primers for amplification of the sex - informative segment of amelogenin intron 1, yielding PCR products differing in size 6 nucleotides between amelogenin locus on chromosome X and chromosome Y. STR typing of Y-chromosomal STRs was performed using the PowerPlex Y System (Promega, Medison, WI, USA) and the PowerPlex Y23 System (Promega). Both kits contain the same twelve microsatellite markers, whereas the PowerPlex Y23 contains eleven additional STRs. Overall, 23 STR loci were amplified on Y chromosome. The amplification protocols and the thermal cycling conditions for all four PCR reactions were according to the manufacturer's instructions.[30–33] The Nexus Master Cycler (Eppendorf, Hamburg, Germany, EU) was used for the amplification of aged DNA. The fluorescent-labelled PCR products were separated on an automatic ABI PRISM™ 3130 Genetic Analyzer (Applied Biosystems) using the 3130 Performance Optimized Polymer 4 (Applied Biosystems). The genetic profiles were determined using the Data Collection v 3.0 and GeneMapper ID v 3.2 (Applied Biosystems) software with a 50 relative fluorescence units (RFU) peak amplitude threshold for all dyes. STR typing was also carried out for persons who were included in the elimination database and for reference sample using the NGM kit (Applied Biosystems) and the PowerPlex Y23 kit (Promega). Genetic profiles from elimination database were compared with those obtained from the cranium to monitor possible contamination of aged DNA with contemporary DNA.

3. Results and discussion

3.1. Anthropological analysis

The analyzed cranium is shown in Fig. 1. The anthropological analysis revealed an overall small cranium, but with evident muscle attachment sites (Fig. 1a).

Despite an insignificant glabella, other sexually dimorphic traits (i.e. a robust mastoid process with a developed supramastoid crest, a moderately prominent nuchal crest) spoke in favor of a male individual (Fig. 1a and b). The first analysis of the cranium conducted after excavation, did not arrive to any conclusion regarding the sex of the cranium due to ambiguous sexual features, which also encouraged the current genetic analysis aimed to identify the missing mother of a woman. Sexing human remains from a cranium is less exact than when relying on the complete skeleton or the pelvis. Population affiliation, age, secular changes as also the experience of the observer can affect the correct assessment of sex in human remains.[34–36] In this case, the size of the cranium and a small glabella, cited as one of the most reliable cranial sex indicator,[34,37,38] could have confounded the investigators that did not focus on other discrete sexually dimorphic cranial traits, like the developed mastoid process and nuchal region. Problems of sexing human remains when relying on some of the “most reliable” cranial dimorphic features have already been noted during the identification process of human remains from the Balkan region. Namely, usual sex indicators had little success in sexing male remains.[39]

The morphological analysis of the cranium revealed many features characteristic of Caucasoids, like a narrower nose opening, a sharp bony ridge at the inferior nasal margin, a prominent anterior nasal spine, a narrow maxilla, oval orbits with a smaller interorbital distance and the presence of malar tubercles along with non projecting zygomae. The European ancestry was confirmed also by metrical data in the program AncesTrees (with an accuracy of 82% when tested against Asian and African populations).[40]

The age of the individual was approximated based on the dental development staging and cranial sutures state.[41] The second left maxillary molar has erupted and fully developed. The left maxillary third molar has not fully erupted. When extracted, it showed a clear