BABEȘ-BOLYAI UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

Summary

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Alexandra GÎNGUȚĂ

Cluj-Napoca
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Unlocking maternal relationships of Gepids and German Saxons from Transylvania by ancient DNA analysis

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List of abbreviations

aDNA – ancient DNA

bp – base pairs

*F*st – Fixation index

Hg – haplogroup

HVR – Hypervariable Region

MDS - Multidimensional Scaling

mtDNA – mitochondrial DNA

mtHg – mitochondrial haplogroup

nDNA – nuclear DNA

NGS – Next Generation Sequencing

PCA – Principal Component Analysis

PCR - Polymerase Chain Reaction

rCRS – Revised Cambridge Reference Sequence

SHD – Shared Haplogroup Distance

SNP – Single Nucleotide Polymorphism

Key words: ancient DNA, mitochondrial DNA, human population genetics, Transylvania, historical populations, NGS

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Preface

The present doctoral thesis has an interdisciplinary character by addressing Transylvanian history from genetic perspective. The novel science brought by this thesis is based on the first mitogenomes assembled from Gepids, as well as the unprecedented recovery of mitochondrial DNA (mtDNA) data from Transylvanian German Saxons, historical populations that left their marks on the historical Central-Eastern Europe, in general, and Transylvanian region, in particular.

This work is comprised of two distinct studies and it is the outcome of three years of molecular biology-oriented research (though augmented by archaeological knowledge) performed at two institutions in Romania and Hungary. Thus, the first research, described in Chapter III of this thesis, was performed at the Department of Archaeogenetics of the Institute of Hungarian Research and the Department of Genetics, University of Szeged, Hungary, under the supervision of Assoc. Prof. Dr. Tibor Török. The second study, detailed in Chapter IV of the present thesis, was conducted at the Interdisciplinary Research Institute on Bio-Nano-Sciences, "Babeș-Bolyai" University, Cluj-Napoca, Romania, under the supervision of Assoc. Prof. Dr. Beatrice Kelemen. Besides, the doctoral stage and, implicitly, the thesis, benefitted from the formal guidance (and scientific input) of Prof. Dr. *Habil* Horia-Leonard Banciu (Faculty of Biology and Geology, "Babeș-Bolyai" University, Cluj-Napoca, Romania) and Prof. Dr. Sorin Nemeti (Faculty of History, "Babeș-Bolyai" University, Cluj-Napoca, Romania). Noteworthy, Prof. Dr. Sorin Nemeti supervised the sampling and dating of the provided samples, as well as the archaeological background and the historical interpretation of the investigations.

Outline of the thesis

Ancient DNA (aDNA) offers a special way to track genetic history across time. In order to better understand the evolution of humans, the analysis of mitochondrial DNA sequences proved to be a powerful tool, providing sufficient molecular resolution to discern evolutionary patterns that developed over millennia. We attempted to utilize the information hidden in aDNA to investigate for the first time the genetic ancestry of two historical groups from Transylvania, a Gepid and a German Saxon group, providing insights into their origin and revealing relationships with other ancient and modern populations.

The aims of this doctoral thesis were:

a. To explore for the first time the genetic diversity of a Gepid population from the 5^{th} century CE and a German Saxon population from the 12^{th} century CE from Transylvania, Romania, by using information provided through ancient DNA.
b. To evaluate the connections between the investigated groups and other ancient and modern populations available in the public databases.
c. To provide insights into the demographic and admixture events of the ancient populations from the perspective of the maternal lineages.

The thesis begins with **Chapter I**, which outlines a few basic principles of population genetics, fundamental information about aDNA and what is known so far about the European populations based on the mtDNA and whole genome data.

Chapter II provides a concise historical context of the region and the considered populations in order to portray a consistent image, contoured by various scientific fields, also supplemented with information about the cemeteries. **Chapter II** aims to describe the cultural

identification of the studied populations based on historical records and archaeological findings. By combining cultural and genetic indicators, we could determine whether or not cultural uniformity conceals a distinct genetic background.

The aim of the study detailed in **Chapter III** was to get clues on origins of elusive Gepid population in Transylvania and its connections to other ancient and modern Eurasian people. To address this goal, we assembled 46 full mitogenome sequences from 3 Gepid cemeteries from Transylvania. The retrieved mitogenomes were unmatched in the known literature for a Gepid group and allowed meaningful insights into their population genetic structure based on the maternal lineages.

In **Chapter IV** we aimed to examine the maternal genetic diversity of a Middle Age German Saxon population in Transylvania and asses its relations to other ancient groups, as well as to succeeding Eurasian populations. To achieve this aim, we recovered and analyzed 13 sequences of the hypervariable regions of the mitochondrial genome from medieval individuals excavated in the Feldioara Necropolis, Brașov County. Based on historical and archaeological records, they may have been members or decedents of a population that reached the farthest German colonization limit in the Middle Ages.

Chapter V provides an overview of the key scientific contributions of this thesis, formulates the general concluding remarks and asserts future perspectives based on the experimental findings.

The focus of this work is Transylvania, a major historical location that, over the years, has been ruled by diverse entities and has witnessed interactions between various communities. Today, Transylvania retains a distinct identity due to the many political pressures that share diverse ideas and have the potential to interfere with scientific discussions. The study encourages the research of the population diversity by bringing new genetic data from the Migration Period and Middle Ages, which are the most underrepresented periods concerning the number of ancient individuals that were genetically investigated from Romania.This research delivered the first genetic data about two historical groups from Transylvania, investigated their maternal genetic diversity and determined similarities and differences from other ancient and modern populations. The results presented in this thesis will help illustrate a part of the genetic structure of the ancient people inhabiting Transylvania, as well as reconstructing the past genetic history of Eurasia, including migration events and admixture.

CHAPTER I: Ancient DNA analyses reveal migrations and admixture of ancient populations

I. 1. Fundamentals and approaches of learning population genetics

Population genetics is a branch of genetics that explains the evolutionary changes in populations by studying the change of the genetic composition of organisms over time (Relethford, 2012). By studying the genetic material of an organism, we can reveal the evolutionary history of individuals or populations, because genomes are records of evolutionary changes. Genetic variation in populations is the result of various processes like geographical isolation, founder events, admixture and migration (McVean, 2009).

One of the most common analyses methods in population genetics is Principal Component Analysis (PCA). It is a statistical method that uses datasets with large numbers of variables or dimensions and reduces them to a few principal components that explain the major variability of the data (Reich *et al.*, 2008).

*F*st or the fixation index is other important statistical method used for population differentiation, which can be estimated from SNP data. *F*st is the proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance. *F*st values are

between 0 and 1, small *F*st values indicate low level of differentiation among subpopulations, while high values indicate high divergence between populations (Holsinger and Weir, 2009).

Multidimensional Scaling (MDS) is a data analysis technique used to explore the structure of similarity between multidimensional data applying dimension reduction. It is a general approach for graphically representing relationships between objects in the multidimensional space (Graffelman *et al.,* 2018).

Shared Haplogroup Distance (SHD) is a novel approach developed by (Maróti *et al.*, 2018) to calculate population genetic distances and study their admixture history. The method also considers the mtDNA genome mutation and fixation rate allowing some relationship between progenitor and progeny haplogroup (Hg) lineages.

Phylogenetic trees are diagrams that represent evolutionary relationships among organisms. The branches in a phylogenetic tree illustrate how groups developed from a number of common ancestors. One of the most popular methods for constructing phylogenetic networks from recombination-free DNA data, like mtDNA data, are Median Joining (MJ) networks (Bandelt *et al.*,1999).

I. 2. Ancient DNA

Ancient DNA is defined as DNA obtained from ancient specimens. It is the most informative biomolecule that can be recovered from biological remains which helps understanding the past. Studying aDNA offers a unique perspective of past times, by revealing the genetic history of extinct organisms and past populations. It represents, in fact, short fragments of an organism`s DNA that began to deteriorate after its death, with several characteristic alterations, hence aDNA studies apply a system of methods with the objective of extracting and recovering the DNA sequences. The information obtained through aDNA studies assist many fields apart from biological sciences, such as anthropology, archaeology and forensics.

I. 2. 2. Technical challenges in aDNA studies

I. 2. 2. 1. Degradation

After the death of an organism, the cellular repair mechanisms cease to function and DNA begins to be degraded by enzymes, as the cellular compartments that sequestered the enzymatic systems break down. DNA is also exposed to external microorganisms such as bacteria and fungi that feed on macromolecules and further degrade them and besides, macromolecules, undergo biochemical degradation processes such as hydrolysis, oxidation which led to breakages in the DNA strand an irreversible loss of nucleotide sequence information (Dabney *et al.*, 2013).

Better understanding in the preservation of ancient biomolecules and screening methods for detection of molecules in bones would allow for a better selection of biological materials.

I. 2. 2. 2. Contamination

Another major challenge in aDNA studies is sample contamination, which greatly challenge the authenticity of aDNA results (Sampietro *et al*., 2006; Dabney *et al*., 2013).

To overcome contamination, strict criteria must be followed when working with ancient remains (Cooper and Poinar, 2000). These will not guarantee that the recovered sequences are authentic, so researchers must always assess the quality of their results when handling aDNA.

I. 3. Ethical considerations

Prior to beginning of any project that comprises destructive analyses there are some ethical concerns that need to be evaluated. aDNA research encompass ethical issues in some of the steps, such as conceptualization, sampling and communication (Orlando *et al.*, 2021; Pálsdóttir *et al.*, 2022). Sampling and analysis of the osteological materials is most of the cases destructive and conservation of the remains needs to be prioritized, because the source of the samples in aDNA studies is unique and irreplaceable.

I. 4. Petrous bone in aDNA analyses - a special case

The petrous bone is located in the temporal region of human skull and it is one of the densest bones in the human body (Chmielewski *et al.*, 2020). Petrous bones are now the priority choice for aDNA studies, as several reports showed that aDNA is preserved better in petrous bones than in other skeleton materials (Pinhasi *et al.*, 2015, 2019; Hansen *et al.*, 2017).

I. 5. Mitochondrial DNA and ancient population studies

I. 5. 1. Mitochondrial DNA – general aspects

Mitochondria are dynamic intracellular organelles present in all nucleated mammalian cells. MtDNA is one of the most important tools used in determination of the origin and genetic composition of different populations and population genetics (Sato *et al.*, 2011; Vai *et al.*, 2015; Csákyová *et al.*, 2016), but it is also used in other fields such as anthropology, medical genetics, forensic science.

MtDNA is preferably targeted in genetic studies of ancient populations because it has a set of distinguishing features from the nDNA. First, mtDNA is present in high copy number in each cell compared to nDNA, which confers mtDNA a higher change of being recovered from the biological remains as it is more likely to survive for prolonged periods than nDNA. Second, it has uniparental inheritance and lacks recombination. MtDNA is maternally transmitted (Giles *et al.*, 1980), and the lack of recombination allows for determination of maternal family relationships. Third, it has high mutation rate, thus mitochondrial genes evolve faster that nuclear genes (Brown *et al.*, 1979). Forth, a human reference sequence was available since 1981 (Anderson *et al.*, 1981), which allowed population studies to expand rapidly.

I. 5. 2. Mitochondrial haplogroups

Studies of mtDNA from different human populations identified several continent specific stable polymorphic sites, which are called haplogroups (Torroni *et al.*, 1996). Haplogroup members share one to several distinguishing mutations descended from the same ancestral mtDNA molecule (Torroni *et al.*, 2020). Human mtDNA variations are recorded by aligning the mitochondrial sequences to the revised Cambridge reference sequence (rCRS) (Andrews *et al.*, 1999).

The mtDNA haplogroup nomenclature is provided by PhyloTree (phylotree.org). PhyloTree is a comprehensive phylogenetic tree reconstructed from the worldwide human mtDNA variations. It comprises more than 5,400 subhaplogroups with their defining mutations, giving a detailed genetic view of the evolution of humankind from a matrilineal perspective (van Oven and Kayser, 2009).

I. 6. Genetic history of Europe reconstructed from mitochondrial and whole genome data

The genetic diversity of modern Europeans was profoundly influenced by several major events that took place during our history. The Neolithic revolution represents the beginning of major changes in human history. The Neolithic transition had the greatest effect on the demographic process that shaped the European gene pool by succession of migrations phases and local admixture (Lazaridis *et al.*, 2016). Modern Western Eurasians show a large degree of genetic homogeneity, which indicates extensive migrations and admixtures between the people of Europe, and the neolithic transition was one of the biggest mechanisms for this homogenization. The Balkan populations, including Romanians, acquired most of their genetic variation from Middle-Late Neolithic migrations spreading from Anatolia into the Balkan Peninsula and Central Europe (Hervella *et al*., 2015).

Recent studies that focused on the genetic transformation of Europe have shown that most present-day Europeans derive from at least three highly differentiated populations, or three ancestral Paleolithic components.

I. 7. Genetic data available from human populations in Romania

The ancient territory spanning modern Romania area housed major crossroads between Europe and Asia, which were used by migrating populations between the two continents throughout history.

Several studies assessed the genetic diversity of the modern Romanian populations showed that the mitochondrial gene pool is geographically homogeneous across the country, while the haplogroup composition shows signs of admixture between populations of diverse origin. Compared to other regions of Romania, Transylvanian samples have a higher affinity to Central European populations, which can be attributed to the migratory routes influenced by the Carpathian Range, while the other three regions rather cluster together with Slavic populations (Cocoș *et al.*, 2017). The genetic resemblance of Transylvania to the populations of Europe can be explained by the higher admixture over time of Transylvanian people with Western European groups.

Conclusions

Ancient DNA data is still unevenly available from different regions and periods, but from the territory of Romania some of the most important archaeological discoveries were made, including the evidence of the earliest human presence on Europe. Despite the lack of aDNA data from the majority of periods, ancient data collected from the historical areas of Romania reveal distinct European and Asian influences, indicating a mixed ancestry of the studied populations. In the future, a larger dataset is needed for better understanding the processes of admixture between ancient populations living in that area.

CHAPTER II: Archaeological and historical view of the populations used in this study

II. 1. Introduction

In the attempt to reconstruct history, genetic data must always be evaluated together with other evidences from historians, archaeologists, anthropologists, and linguistics. In such a multidisciplinary approach, genetic data can be the most informative to reveal the origin and relationships of past populations. Probably due to disciplinary considerations, there is yet a strict separation between the social and biological sciences. The relevance of the interdisciplinary dialogue resides in tackling these boundaries and identifying common ground, as we tried to achieve in this study about the ancient populations from the Transylvanian territory. With the help of genetic interpretations, we tried to bring further evidence in understanding the origin of two ancient groups, namely the Gepids and German Saxons from Transylvania and integrate the results into a historical perspective.

II. 2. The particularities of Transylvanian historical populations

It is quite challenging to reconstruct the movements and civilizations because so many different populations left their marks here and the land belonged to multiple modern states. This is also true for migratory populations including barbarian ones.

Transylvania is one of these regions that, due to political debates between different modern countries, each used the archaeological records to construct the national discourse. For studying the Migration period and Early Middle Ages, where many written sources are

reporting mass migrations, many particularities are explained by migration applying mixed argumentation, and these interpretations are not free of political ideology (Dobos, 2021).

II. 3. Historical and archaeological background of Gepids

The study of the barbarian groups, so far, focused mainly on Goths, Franks, Anglo-Saxons, Huns, Alamanni and Lombards, and recently on Vandals and Slavs.

Concerning the barbarian Germanic groups, among which Gepids belong to, the latest comprehensive monograph was written in 1955, *Die Gebiden*, where it is encompassed more than two centuries of the history of the Carpathian Basin (Kiss, 2014). Gepids were one of the East Germanic people who lived in the Carpathian Basin. Unfortunately, there are limited archaeological sources and interpretations for one to precisely establish the history of the Eastern Germanic tribes in the Carpathian Basin. Gepids are mentioned in the 5th century CE migrations, together with the Huns, and after the death of Attila in 453 CE and the dissolution of the Hunnic Empire, Gepids took over the territories east of Tisa, including Transylvania (Stanciu, 2010) and became a dominant force, expanding their territories to Sirmium and its surroundings (Kiss, 2014).

In the middle of the 6th century CE, the expansion of the Gepidic Kingdom was halted and defeated by The Byzantine Empire and Longboards.

II. 4. Gepids traces on the actual territory of Romania and the row-grave cemeteries

The interpretation that Gepids were present in the Upper Tisa region in the early 4th century CE is influenced by Jordanes' story which talks about a conflict between the Goths and the Gepids in the 3rd century CE in an area "hemmed in by rugged mountains and dense forests", but later discoveries suggest that the indicators of early Gepid culture are not always part of a uniform archaeological group (Dobos, 2019). Several findings dated in the 5th century CE, indicate a Germanic center of power active in the first half of the 5th century CE in Northwestern Romania, most likely attributed to Gepids.

For the Transylvania region, the exact date when it was occupied by Gepids cannot be stated because there are no historical or archaeological data that can support Gepid presence earlier than the middle of the 5th century CE. The presence of the row grave cemeteries, which are connected with Gepids` culture, can be seen in Transylvania from the late 5th century until the first half of the 7th century CE.

II. 6. Historical and political context of the German settlement in Transylvania during the Middle Ages

Starting with the 10th century CE, the Intra-Carpathian Arch was conquered by the Hungarian Kingdom and in the years to follow, it brought various people to Transylvania, including Pechenegs, Cumans, Szeklers, and Saxons. The main factor that initiated the migration of Germanic people in Transylvania were the crusades.

Saxons, a Germanic group, were the last people who settled in Transylvania in the Middle Ages (Nägler, 1981). Their arrival occurred in the 11th century CE and was a result of German colonization efforts in the Eastern Carpathian Basin. The first Germanic people settled in the Hungarian Kingdom probably during the ruling of Ladislaus I or Saint Ladislas (1077-1095). During the ruling of King Coloman (1095-1116) they are proclaimed *liberi et hospites* (Nägler, 1981) and they were given many privileges and lands.

II. 7. Archaeological background of the studied cemeteries

The location of the studied cemeteries is shown in **Figure II_1**.

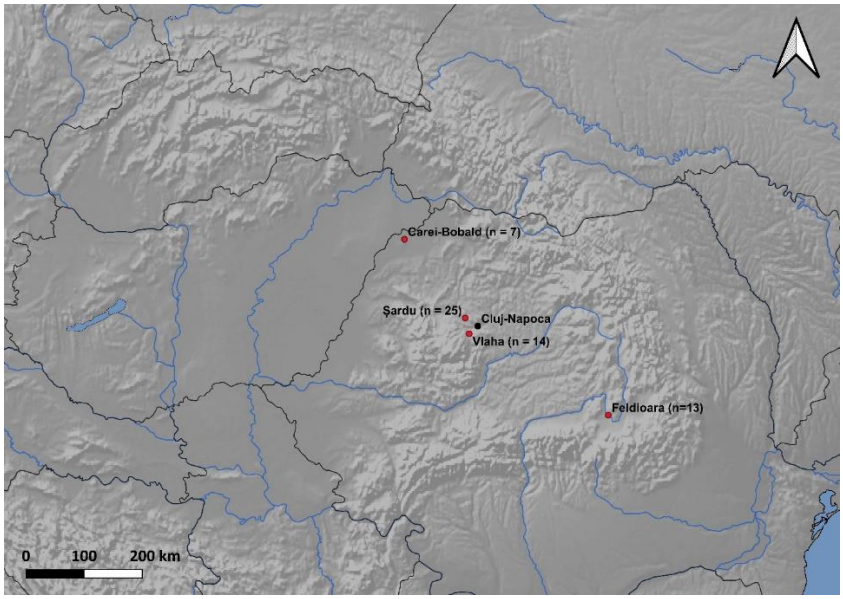

Figure II_1. Location of the cemeteries sampled in this study.

II. 7. 1. Carei–Bobald (Satu Mare County)

The archeological site is situated close to the Hungary border in Northwestern Romania. Geographically, the Gepid settlement of Carei indicates the northeastern boundary of the Gepid settlements from the Tisa Plain. The excavations uncovered 18 Gepid features, 10 Bronze Age features, 16 features of Celtic date—of which 2 pottery firing workshops, 24 Gepid period burials and 27 features from 16–17th century. 24 inhumations from the Gepidic cemetery were investigated. Most graves are consistently aligned from west to east. They are included in the row-grave burial horizon for the 6th century CE due to both their orientation and parallel row organization. Based on the discoveries, Carei–Bobald settlement and cemetery became the reference point for the Tisa Plain area, by the northern border of the Gepid finds.

II. 7. 2. Șardu (Cluj County)

The archaeological site is situated in the medieval province of Transylvania, in Central-Western Romania. The tombs were approximately placed in rows that were north-south oriented, with some groupings containing 2-4 graves. The funeral rite also contains inhumation in graves with a west-east orientation. First impressions suggested that the cemetery was in use during the Gepidic Age, based on the artifacts and burial practices during the second half of the 5th century–first two thirds of the 6th century CE.

II. 7. 3. Vlaha/Magyarfenes-Pad (Cluj County)

The archaeological site of Vlaha/Magyarfenes-Pad is located in Northwestern Transylvania, close to Cluj-Napoca city, on the site of the former Roman city, Napoca.

An area of 1.1 ha was completely investigated, with 1340 archaeological structures—including 308 tombs from the Gepid period—being found and explored. Settlements from the Late Bronze Age and Early Iron Age II (Ha B2), were located on the same site.

The Vlaha Necropolis is a member of the eastern group of necropolises that are "arranged in parallel rows of tombs," (Reihengräberfelder). Their appearance in Transylvania can be dated to the transitional period between the 5th and 6th centuries CE. The Vlaha cemetery can be dated to the first two thirds of the 6th century CE based on archaeological findings.

II. 7. 4. Archaeological site from Feldioara (Brașov County)

The Feldioara archeological site comprised levels from the Neolithic era, early Bronze Age, first Iron Age, second Iron Age, Roman, Medieval, Premodern and Modern Eras.

The first major archaeological excavation at Feldioara was carried out between 1990-1995 and the second in 2013-2017, prior to the restoration of the settlement. (Ioniță, 2004).The research was presented in a number of publications.

II. 7. 4. 1. Medieval necropolis from Feldioara (Brașov County)

The archaeological excavations unearthed s number of 127 graves, from the estimated number of approximately 200 (Ioniță, 2004). The deceased adults were probably wrapped in shroud and were placed without coffin in pits with a step and with a niche for the head, in supine position, with the head to the west and the upper limbs arranged along the body.

Analogies of the necropolis imply that it belonged to the first wave of German settlers who came to Transylvania after the middle of the 12th century CE. Tombs similar to those from Feldioara were discovered in the area of German colonization in Southern Transylvania from the 12th century CE in Drăușeni (Brașov County), Mediaș and Huet Square from Sibiu (Sibiu County), Orăștie (Hunedoara County) and Sighișoara (Târgu Mureș County) (Ioniță, 2004).

Conclusions

From historical sources and archaeological studies alone, it is difficult to recreate the migrations and population admixture of the Migration Period and the Middle Ages in Transylvania. Furthermore, Transylvania was one active region for populations movements and migrations, giving rise to numerous political arguments between modern states in reconstructing its history. Populations chosen for performing the molecular analyses are well dated historically and archaeologically, with specific indicators such as burial ritual and grave goods, that define the time period and culture of Gepids and German Saxons from Transylvania.

CHAPTER III: Maternal lineages of Gepids from Transylvania

Results presented in Chapter III were published in Gînguță, A.; Kovács, B.; Tihanyi, B.; Maár, K.; Schütz, O.; Maróti, Z.; Varga, G.I.B.; Kiss, A.P.; Stanciu, I.; Török, T.; Neparáczki, E. Maternal Lineages of Gepids from Transylvania. *Genes* **2022**, *13*, 563. https://doi.org/10.3390/genes13040563

II. 1. Introduction

According to historical records, Gepids were a Germanic tribe that arrived in the Carpathian Basin during the Migration Period. Gepids formed alliances with the Huns, and following the fall of the Hun Empire, they formed a kingdom, known as the Gepid Kingdom.

This migratory group had a significant impact on the history of Central Europe, yet little is known about them because no modern European nation claims their legacy. The European migration period is still underrepresented genetically, with only a few samples from populations associated with Avars (Csosz *et al.*, 2016; Šebest *et al.*, 2018; Neparáczki *et al.*, 2019; Csáky *et al.*, 2020; Maróti *et al.*, 2022), Lombards (Alt *et al.*, 2014; Vai *et al.*, 2015, 2018; Amorim *et al.*, 2018; Poma *et al.*, 2019), Goths (Stolarek *et al.*, 2019), and Huns (Neparáczki *et al.*, 2019) available. Prior to this study, there was no genetic data available from the Gepid period.

All sampled cemeteries featured burials and grave items associated with the Classical Gepidic Period. Our objective was to examine the genetic composition of the Gepid period

population from Transylvania in order to determine its origin, demographic organization, and connections to other ancient Eurasian populations.

III. 2. Materials and methods

III. 2. 1. Identification and details about the samples

Samples used in this study and the archaeological background of the cemeteries were provided by I. Stanciu from the Institute of Archaeology and Art History, Romanian Academy, Cluj-Napoca, Romania, A. Dobos from National Museum of Transylvanian History, Cluj-Napoca, Romania and R. Gindele from County Museum in Satu Mare, Romania.

Each sample has an identification number comprised of the name of the cemetery and the number of the grave or the number of the archaeological complex. Osteological samples were taken from the burial sites with good state of preservation, both female and male graves, adults and infants and from graves with rich inventory and with no inventory at all.

III. 2. 2. Sample preparation

Sample preparation, DNA extraction, library preparation, low shotgun sequencing and mitochondrial enrichment were performed in the sterile laboratories dedicated for aDNA work at the Department of Archaeogenetics of the Institute of Hungarian Research and Department of Genetics, University of Szeged, Hungary.

Teeth were cleaned with bleach and Milli-Q ultra-pure water, followed by ultraviolet exposure. Temporal bones were cleaned by tapping each side with a bleached soaked napkin, followed by ultraviolet exposure.

III. 2. 3. DNA extraction

DNA extraction was performed in a PCR free room, separate from the sample preparation facility. In case of teeth, a minimally destructive protocol which preserves the integrity of the teeth (Harney *et al.*, 2020) was used.

In both teeth samples and pars petrosa powder samples, a pre-digestion step was performed. Quantity of DNA extracts were measured with Qubit 3.0 Fluorometer (Invitrogen) using the dsDNA High Sensitivity Assay kit, using 1 µL of DNA extract.

III. 2. 4. NGS library preparation

aDNA library preparation protocol used in this study requires the production of only one library per each sample and uses a partial UDG treatment of the libraries to reduce the mismatches the aDNA sequence, while preserving some errors to allow for the assessment of the authenticity of ancient molecules (Rohland *et al.*, 2015).

III. 2. 5. NGS Low Coverage Shotgun Sequencing

The estimation of the endogenous human DNA content of the libraries were performed on the Illumina iSeq 100 platform.

III. 2. 6. Mitochondrial DNA capture and enrichment

Mitochondrial DNA was captured from pooled sequencing libraries of multiple individuals using PCR products or DNA baits. Hybridization mixture was prepared based on the endogenous DNA content and DNA concentration of the samples. Samples with closest endogenous concentration were grouped together.

III. 2. 7. Mitochondrial genome sequencing

The enriched mitogenome libraries were sequenced to collect the mitochondrial information of the individuals. After the mitochondrial enrichment, DNA was quantified by Qubit 3.0 Fluorometer (Invitrogen). The fragment distribution of the samples was checked with Agilent 2200 TapeStation Genomic DNA High Sensitivity ScreenTape. The sequencing was performed on the Illumina iSeq 100 system.

III. 2. 8. Data analysis

The adapters from the paired-end reads had been removed Only sequences longer than 25 nucleotides were used in the subsequent study. Samples were mapped to the GRCh37.75 human genome reference sequence, which contains the rCRS, (NC 012920.1).

aDNA damage patterns were evaluated to account for post-mortem damage. Sequences were displayed in Integrative Genomics Viewer v1.10.0 to detect possible SNP calling problems. Schmutzi algorithm (Renaud *et al.*, 2015) was used to estimate contamination. Each variant call was inspected manually using the FASTA format in Integrative Genomics Viewer (Robinson *et al.*, 2011).

III. 2. 9. Haplogroup assignment and sex determination

MtHgs were determined using HaploGrep2 (v2.4.0) (Weissensteiner *et al.*, 2016). Two methods that operate with shotgun sequencing reads were used in the determination of the biological sex of the individuals. One is based on the X/Y ratio (Skoglund *et al.*, 2013) and the other one on the endogenous DNA assigned to autosomes (Mittnik *et al.*, 2016). The raw nucleotide sequence data of the 46 Gepid mitogenomes were deposited to the European Nucleotide Archive (http://www.ebi.ac.uk/ena, accessed on 21 February 2022) under the accession number: PRJEB50517.

III. 2. 10. Phylogenetic study

The sequences were aligned using MAFFT version 7 (Katoh *et al.*, 2002; Katoh and Standley, 2013) and median joining (MJ) networks were constructed (Bandelt *et al.*, 1999). Phylogeographic connections were inferred from the geographic origin of the closest matching samples from the database.

III. 2. 11. Population genetic analyses

The mtDNA distribution of the examined samples was compared to populations from an archaic database containing 4,324 ancient Eurasian mitogenomes (Maár *et al.*, 2021), in order to ascertain the genetic distances between ancient populations. We formed 92 groups based on time range and archaeological evidence.

All Gepids samples were combined into a single population for the population genetic analysis. We used three independent methods for determining the genetic similarity of Gepids to other ancient populations (PCA, pair-wise population differentiation values (*F*st), SHD values).

III. 3. Results and discussion

III. 3. 1. Shotgun data

Endogenous DNA content of the extracts varied between 0.1% and 73%. The average DNA fragment size was between 47 and 123 bp. We obtained 38 mitogenomes above 5x coverage and 8 mitogenome with less than 5x coverage. Most of the samples had negligible contamination (0–2%).

III. 3. 2. Haplogroup composition and phylogenetic analysis

The 46 samples belonged to 36 Hgs and 37 different haplotypes. Gepid sequences have the following haplogroup frequencies: H 38%, HV 5%, I 8%, J 4%, N 3%, T 20% (T1 5% and T2 15%), U 11% (U3 3%, U 4 5% and U5 3%).

The Transylvanian Gepid population belonged to Western Eurasian lineages, the majority of which were typical of Northwestern European regions, as evidenced by the phylogenetic trees. Our findings are consistent with the Germanic people historically documented geographic origin in Europe.

Some lineages had the closest parallels from Late Chalcolithic Turkey and Bronze Age Anatolia, suggested Near Eastern affinity. Most likely, this liking for the Near East can be attributed to early European farmers (Haak *et al.*, 2010; Fu *et al.*, 2012). The H6a1b is the only lineage demonstrating Eastern Eurasian affinity and likely immigrated with eastern immigrants during the Migration Period. Haplogroup H6 is mostly found in Near East and Southern Caucasus, with low frequencies in Europe.

Phylogenetic trees also showed that many of the lineages had been present in the Carpathian Basin and the surrounding area from the Neolithic to the Middle Ages. This may reveal a possible population continuity of the 6th century population with earlier medieval

groups in Transylvania. The Gepid group is most similar to Bronze Age inhabitants in the surrounding areas and has a predominance of Central-Northern European heritage.

III. 3. 3. Kinship in the Gepid cemeteries

In all three cemeteries, we found numerous identical haplotypes, which may indicate direct maternal relation between graves that are close together or distant. We discovered three pairs of individuals with the same haplotype in the Vlaha/Magyarfenes-Pad cemetery. The possibility that these people are related increases due to the low frequency of these haplogroups.

In the Șardu cemetery, the results showed a group of four individuals and another two pairs with the same haplotype. The graves' close proximity to one another suggests that a small group of related people were buried there.

Two identical haplotypes from nearby graves were detected in the Carei-Bobald cemetery. Due to the graves position and the nature of the cemetery, these individuals are also very likely related.

III. 3. 4. Population genetic analysis

We merged all of the Gepid sequences from into a single population and calculated their genetic distances from all other ancient populations that were available in the public databases.

PCA obtained from the major Hg frequencies (**Fig. III_1**) shows a cluster of the European populations, regardless of geographical region or period.

The group under study is clustered among European populations, closest to European Neolithic, Copper Age, and Iron Age groups.

Results from the *F*st and SHD analyses were also consistent. Each population genetic investigation confirmed the same genetic pattern, demonstrating that early ancient European mitogenomes were the primary sources of the maternal genetic diversity of Gepids.

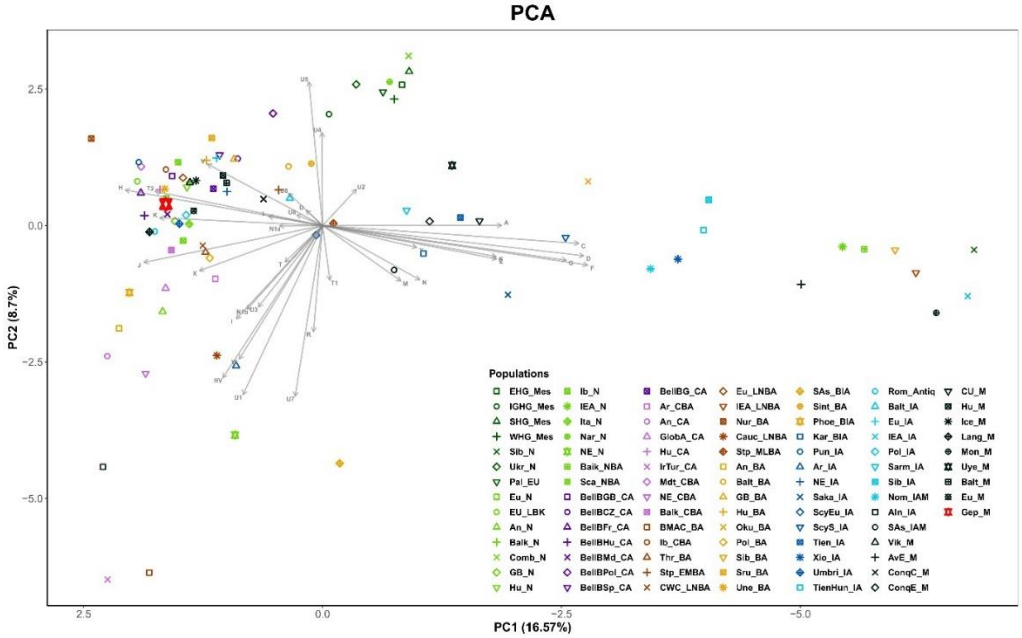

Figure III_1. The principal component analysis computed from the major mtDNA haplogroup distributions of 93 Eurasian populations.

According to population genetic findings, the maternal ancestry of the Gepids may have been primarily derived from indigenous people. This appears to be at odds with historical records that identify the Gepids as Germanic immigrants from Northern Europe, but these records do not rule out the possibility that many of the people could have immigrated from that region given that both regions shared a similar European maternal gene pool.

Conclusions

For the first time, genetic data of the ancient Gepid people is obtained, giving new insights into their genetic structure. The results indicated the genetic makeup of this population from the late 5th and early 6th century CE defined by cultural indicators connected to the Classical Gepidic period in Transylvania.

The genetic information that was recovered pointed to the presence of Northwestern European ancient mitochondrial lineages in the selected group, along with all population genetic analyses confirming this genetic structure. Results demonstrated that early ancient mitogenomes from Europe were the main sources of the maternal genetic diversity in the Gepid group from Transylvania.

CHAPTER IV: Mitochondrial DNA profiles of individuals from a 12th century necropolis in Feldioara (Transylvania)

Results presented in Chapter IV were published in Gînguță, A.; Rusu, I.; Mircea, C.; Ioniță, A.; Banciu, H.L.; Kelemen, B. Mitochondrial DNA Profiles of Individuals from a 12th Century Necropolis in Feldioara (Transylvania). *Genes* **2021**, *12*, 436. https://doi.org/10.3390/genes12030436

IV. 1. Introduction

In the Middle Ages, Transylvania was a province with a distinct identity within the Kingdom of Hungary, whose structure was gradually integrated between the 10th and the 13th centuries CE. This integration was carried out in stages and primarily involved two historical populations known as the Szeklers and the Saxons. The majority of the Western settlers who colonized Southern and Southeastern Transylvania came from Flanders and Saxony, and they are known today as Saxons.

In order to bring new insights in the maternal genetic diversity into this archaic group, we sampled individuals discovered in the Feldioara necropolis to obtain the sequences of the hypervariable regions of the mitochondrial genome. The aim of this study was to provide genetic data about Transylvanian German Saxons, in addition to analyzing their parallels, differences, and connections to existing and succeeding populations.

IV. 2. Materials and methods

IV. 2. 1. Sample preparation

Molecular analysis of the 13 individuals was performed in dedicated aDNA laboratories at the Interdisciplinary Research Institute on Bio-Nano-Sciences, Babeș-Bolyai University, Romania. The surface of the teeth was decontaminated by washing with bleach, followed by an UV-irradiation.

IV. 2. 2. DNA extraction

Tooth powder from the dentine of the root was collected. A silica-based DNA extraction method for isolating DNA from ancient samples was used (Dabney *et al.*, 2013). 1

IV. 2. 3. PCR amplification

The hypervariable regions of the mitochondrial genome were amplified using a "mini-primer set" strategy (Gabriel *et al.*, 2001). This strategy aims to target smaller amplicons and increase specificity so that more authentic DNA sequences can be extracted from ancient remains.

IV. 2. 4. Molecular cloning, sequencing and data analysis

PCR amplicons were cloned using the Sticky-End Cloning Protocol from the CloneJet PCR Cloning kit (Thermo Scientific). The transformation was performed using *Escherichia coli*, strain DH5alpha. For every fragment, 6 clones were Sanger sequenced at Macrogen Europe. The standard primer used for the sequencing was pJET1.2R.

The fasta sequences were aligned with the rCRS (Andrews *et al.*, 1999). Haplogroup was determined using the sequence polymorphisms of the HVR regions, using HaploGrep2 (Weissensteiner *et al.*, 2016), accessed on 22.06.2020.

IV. 2. 5. Population genetic analyses

In order to assess the affinities of the analyzed individuals with other ancient and modern populations, we compared the new control region sequences to all mtDNA sequences found in online databases. For comparing with modern populations, a previously published (Rusu *et al.*, 2018) database containing 35 modern Eurasian populations was used.

Based on the haplogroup frequencies of medieval and modern populations, PCAs and a hierarchical clustering were plotted and the pairwise differences were calculated.

IV. 3. Results and discussion

IV. 3. 1. MtDNA polymorphism of the control regions

The 13 individuals had a significant degree of variability, with 13 distinct haplotypes. The German Saxon group from Feldioara consisted primarily of common Eurasian haplogroups (H, HV, I, J, U4 and U5), except for one individual, who demonstrated genetic contribution from Central Asia. We found no identical maternal lineages.

IV. 3. 2. Population genetics analyses

Regardless of populations affiliation with a particular region, the plot depicts a general clustering of the majority of ancient European groups along the first two PCs **Figure IV_1**. Inferring unique genetic impacts in these two regions divided by the Carpathian Arch, the medieval population from Transylvania (cROU-med) is distantly located from the medieval population from Dobruja.

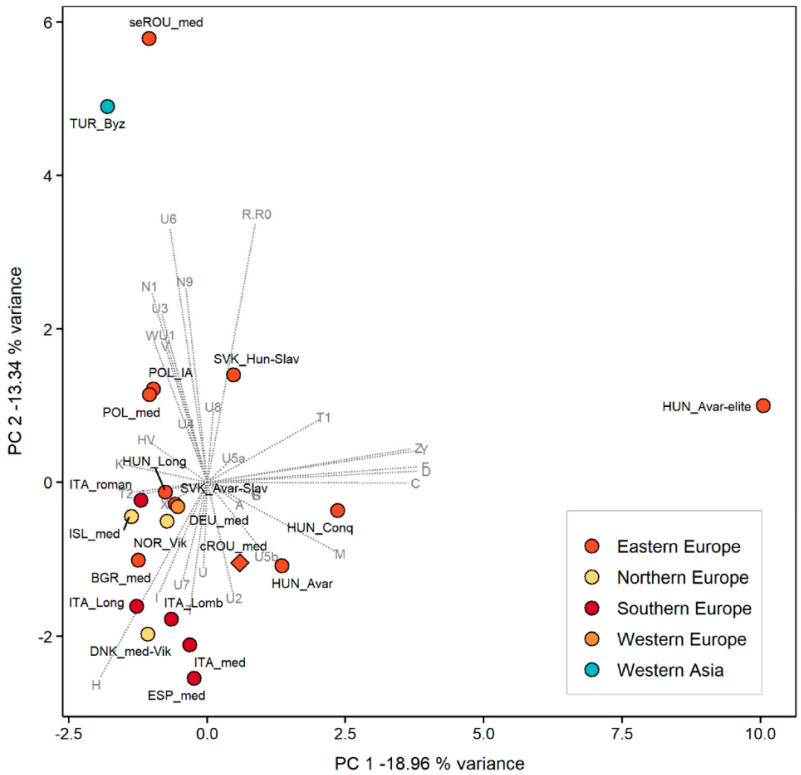

Figure IV_1. PCA plot of the 21 acient populations from Eurasia.

The medieval groups from Southern Europe appear to be more closely clustered among the ancient groups. On the PCA drawn based on mtDNA haplogroup frequencies of modern Eurasian groups (**Figure IV_2),** the Transylvanian population under study is just outside the European populations cluster and had a higher genetic affinity with some Slavic populations (Ukraine and Slovakia) and contemporary groups from the Baltic region (Finland, Latvia, and Estonia).

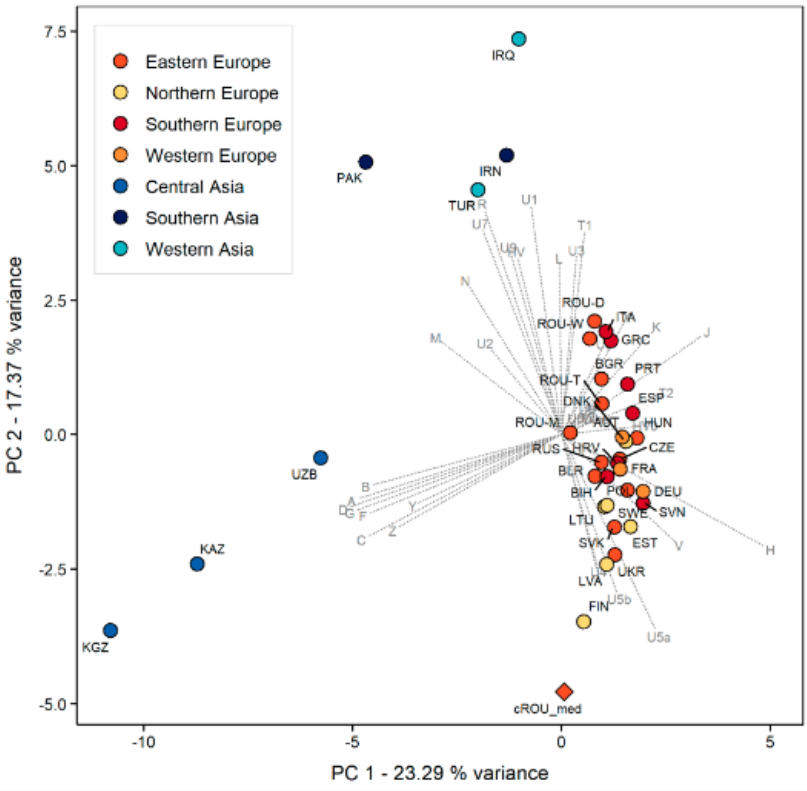

Figure IV_2. PCA plot of the modern populations with two medieval populations from the territory of Romania.

Conclusions

The 13 individuals included in this study possess diverse mitochondrial lineages, indicating a lack of tight maternal relatedness. This level of variety may be indicative of a group that has only recently appeared and has a significant number of members with different racial and ethnic backgrounds. The majority of the samples belonged to the Western Eurasian haplogroups H, HV, J, I, U4, and U5, with the exception of haplogroup C.

CHAPTER V: General conclusions and perspectives

This thesis presents exceptional genetic data from Transylvania, from historical periods that are lacking genetic information. Results pictured partial maternal structure of the investigated groups and possible genetic origin and will be extremely useful in future studies that target the genetic history of Eurasia, including migration events and admixture between populations and helps reconstructing the human past.

Using NGS technology combined with target enrichment for the first time in studying a barbarian population from Romania, we sequenced 46 full mitogenomes (**Chapter III**). The recovered information showed that the predominant maternal lineages were Northwestern European ones. The results imply that the Gepid maternal ancestry may have derived mostly from local residents from the Carpathian Basin.

We analyzed the HVR sequences of the mitochondrial genome of 13 individuals from Feldioara necropolis. The results showed a heterogenous group, comprising mainly Western Eurasian lineages. The high diversity and lack of maternal relationships may be indicative of a group that had only recently established in a particular area and had a significant number of members with diverse geographical backgrounds (**Chapter IV**).

As perspectives, it would be ideal to sample more ancient individuals from the territory of Romania, to infer ancient populations` structure and genetic connections more accurately. Studying Y-chromosome will increase the resolution of the analysis and will reveal new phylogeographical relationships, as well as new migration patterns.

CHAPTER VI: Dissemination of results and fundings

VI. 1. List of publications included in the thesis as chapters

Chapter III

Gînguță A., Kovács B., Tihanyi B., Maár K., Schütz O., Maróti Z., Varga G.I.B., Attila P.K., Stanciu I., Török T. and Neparáczki E. 2022. "Maternal Lineages of Gepids from Transylvania" *Genes* 13, no. 4: 563. https://doi.org/10.3390/genes13040563

Chapter IV

Gînguță A., Rusu I., Mircea C, Ioniță A., Banciu H.L. and Kelemen B. 2021. "Mitochondrial DNA Profiles of Individuals from a 12th Century Necropolis in Feldioara (Transylvania)" *Genes* 12, no. 3: 436. https://doi.org/10.3390/genes12030436

VI. 2. List of publications not included in the thesis

Varga G.I.B., Maár K., Ginguta A., Kovács B., Tihanyi B., Kis L., Váradi O., Kiss P., Szokolóczi D., Schütz O., Maróti Z., Nyerki E., Nagy I., Latinovics D., Török T. and Neparáczki E. An archaeogenetic approach to identify the remains of the Hungarian Kings. Working Plan. *Ephemeris Hungarologica*, 2021. (1.) 2. 333–342. https://doi.org/10.53644/eh.2021.2.333

Varga G.I.B, Kristóf L.A., Maár K., Kis L., Schütz O., Váradi O., Kovács B., Ginguta A., Tihanyi B., Nagy P.L., Maróti Z., Nyerki E., Török T., Neparáczki E. The archaeogenomic validation of Saint Ladislaus' relic provides insights into the Árpád dynasty's genealogy. *J Genet Genomics*. 2022 Jul 6:S1673-8527(22)00179-5. Epub ahead of print. PMID: 35809778. https://doi.org/10.1016/j.jgg.2022.06.008

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