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DOCTORAL THESIS SUMMARY

Maternal lineages of historical populations at the gates of Europe

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Abbreviations

aDNA	ancient DNA
bp	base pairs
BSA	Bovine Serum Albumin
CTAB	Cetyl-Trimethylammonium Bromide
DNA	Deoxyribonucleic Acid
HC	Hierarchical Clustering
HTS	High-Throughput DNA Sequencing
HVS-I	HyperVariable Segment I
HVS-II	HyperVariable Segment II
MRCA	Most Recent Common Ancestor
mtDNA	mitochondrial DNA
NGS	Next Generation Sequencing
np	nucleotide positions
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PCs	Principal Components
PTB	N-phenacylthiazolium bromide
rCRS	revised Cambridge Reference Sequence
RFLP	Restriction Fragment Length Polymorphism
RSRS	Reconstructed Sapiens Reference Sequence
SHA	Shared Haplotype Analysis
SNP	Single Nucleotide Polymorphism

Keywords

Ancient DNA, mitochondrial haplogroups, dual DNA-protein extraction, Next-Generation-Sequencing, genetic diversity, human population genetics

Introduction

The origins and evolution of mankind, as well as its population history, have sparked curiosity for millennia. The human genome is continuously shaped by major demographic events and since the imprints are inherited from previous generations a permanent record of its history is stored in the present-day human genetic material. Therefore, the study of genetic variation in humans is a useful tool to assess migration patterns, populations' genetic response to admixture and populations' origins. While recent demographic changes also have an influence on the current human gene pool and can potentially mask more ancient processes, direct access to DNA from the fossil record and archaeological sources provides highly valuable information for a better understanding of the genetic history of human populations.

I. Ancient DNA as a research tool used to unveil the human past

The research field of ancient DNA has emerged in the '80s and has undergone impressive transformations. Early aDNA investigation mainly focused on extranuclear genomic segments (mitochondrial and/or chloroplast DNA) which are more abundant than nuclear DNA in a cell, facilitating the retrieval of genetic data and the reasonable replication of results. Due to the staggering technical improvement (massive parallel sequencing supported by an increase in computing performance) made in the last decade, it became possible to recover and analyze the nuclear DNA information on a more routine basis (Linderholm, 2016). At the same time, the field has leaped from resolving hundreds to hundred million base pairs (bp), a technological shift that deepened our knowledge of the demographic history of humans (and our closest relatives) (Slatkin and Racimo, 2016).

1. A brief history of the 'ancient DNA' field

The past 30 years have witnessed the publication of increasingly numerous aDNA studies that have covered a broad range of issues in an attempt to test distinct hypotheses used in evolutionary and population genetics studies to reconstruct the past.

1.1. First aDNA sequences

The study field of 'Ancient DNA research' dates back to 1984 when the retrieval of DNA isolated from a 140-year-old museum specimen of the extinct quagga (*Equus quagga*), a relative of horses and zebras, was reported by Higuchi et al. (1984). This seminal study not only showed that DNA molecules can be preserved and recovered from ancient specimens, but also revealed the power of aDNA analysis to address questions regarding the phylogenetic relationships of extinct creatures, as the mtDNA results showed that the quagga was, in fact, more closely related to the plains zebra than to horses (Higuchi et al., 1984; Stoneking, 2016).

One year later, another aDNA study was reported by Paabo (1985) who recovered and sequenced a 3.4-kbp cloned human DNA fragment obtained from an Egyptian mummy dating back to 2,400 years ago. Further research (Paabo and Wilson, 1988) has drawn attention to one of the greatest issues associated to aDNA research, contamination, and it is now accepted that the first genetic data from the above-mentioned mummy specimen were the results of contamination with exogenous DNA.

1.2. Methodological milestones and aDNA key studies

The aDNA field rapidly expanded once the bases of PCR were established (Mullis and Faloona, 1987). Ancient genetic data obtained *via* PCR were used to analyze a varied range of species, with wide temporal and geographical distribution to address various biological questions: phylogenetics and evolutionary processes, population history and phylogeography, diet and behavior, and different aspects of domestication (for a more comprehensive list see reviews of (Hofreiter et al., 2001; Paabo et al., 2004; Paijmans et al., 2013). At the end of the 2000' it was revealed that many of the described aDNA sequences (*e.g.* amber-preserved insects) could not be reproduced (Austin et al., 1997), while other investigated aDNA (*e.g.* from dinosaur bone) turned out to be the result of contamination with exogenous DNA (Young et al., 1995). These early studies illustrated the difficulty of avoiding contamination when working with aDNA.

Almost all limitations associated with PCR-based approaches coupled with traditional Sanger sequencing were overcome in the first decade of the 20th century after the advent of NGS technologies (Margulies et al., 2005). The development of NGS has paved the way for improvements in other methodological applications with crucial implications for aDNA research, such as DNA extraction and targeted enrichment (Hofreiter et al., 2015). The large sequence data generated by NGS also stimulated the development of bioinformatics tools for the reconstruction of the original sequence of a sample from multiple reads.

Using NGS more insights into human evolution and the relationships between modern and archaic humans were gained (Fu et al., 2015; Hajdinjak et al., 2018; Reich et al., 2010) with promises that genetic secrets of even more remains will be successfully deciphered. For example, large-scale studies on dozens or thousands of ancient samples focused on various time transects provide a clearer picture of Eurasian population history (Allentoft et al., 2015; Damgaard et al., 2018; Mathieson et al., 2018; Mathieson et al., 2015; Neparaczki et al., 2018; Stolarek et al., 2018; Tassi et al., 2017; Wang et al., 2018). Even though NGS has revolutionized the ancient DNA field, this is an ongoing research because there are several areas where there might still be room for technological improvements, and more challenges lie ahead.

2. Properties of aDNA and associated challenges

In contrast to contemporary DNA research, the studies on ancient genetic data are subjected to methodological problems that are peculiar to the field and which arise as a result of the aDNA features. The central difficulty is to generate sufficient authentic DNA sequences to make a study conclusive (Gilbert et al., 2005).

2.1. DNA preservation and *post-mortem* modifications

After the organism becomes inactive (dead or dormant), the cellular repair processes cease, and thus the DNA damage accumulates progressively. In addition, the cellular compartments start disintegrating and as a consequence, the DNA molecules become exposed to the intense effects of various factors that affect its stability and may cause the loss of retrievable DNA. Besides the decrease of the total amount of DNA preserved in ancient specimens, different forms of damage can occur in aDNA (strand breaks, oxidative lesions, DNA crosslinks, and hydrolytic lesions). For example, DNA fragmentation that limits the length of the DNA sequences that can be amplified *via* PCR, lesions that block the activity of the DNA polymerase, and lesions that cause incorrect bases to be incorporated during PCR, impose great challenges on the experimental steps used during aDNA recovery (Dabney et al., 2013b).

In addition to the chemical processes that affect the DNA structure and preservation after the death of organisms, the funerary context/storage environmental conditions (humidity, temperature, salinity, and pH) play an important role in the degradation progress (Dabney et al., 2013b). The DNA survival is prolonged under favorable circumstances where the remains are frozen or become desiccated immediately after death (Orlando et al., 2013; Willerslev et al., 2007). The rates of DNA decay differs in distinct types of tissues. The mineralized tissues (bones and teeth) are predominately used as a source for aDNA extraction because the adsorption of DNA to the inorganic fraction of hydroxyapatite is thought to hinder the rate of degradation and because of their ubiquity in the archaeological and paleontological record (Campos et al., 2012; Lindahl, 1993).

2.2. Contamination and results authentication

Contamination is one of the most serious concerns in aDNA studies, being especially pronounced in human/hominin aDNA analysis because of the tight evolutionary relationship between the archaeological human remains and the modern humans handling them from excavation to laboratory procedures (Kirsanow and Burger, 2012). The contamination issue is in fact a problem of relative abundance between the endogenous and exogenous DNA. Even in well preserved archaeological specimens the amount of amplifiable DNA is significantly lower (thousand times) than potential contaminant molecules in the environment (*e.g.* aerosol droplets)

(Willerslev and Cooper, 2005). Human modern contamination is the most insidious type, but there are two other forms of contamination that can affect the aDNA analysis: co-extracted inhibitory substances and environmental microbial DNA (Stoneking, 2016).

During the laboratory procedures, the largest potential source of contamination associated with all PCR-based methods is represented by amplification products from previous experiments (Deguilloux et al., 2011; Kirsanow and Burger, 2012). Therefore, standard strategies were described and employed in order to avoid, minimize and detect contamination (Cooper and Poinar, 2000; Paabo et al., 2004; Poinar, 2003). The most important criteria used to ensure aDNA authenticity include: i) the use of clean, physically isolated area, dedicated to aDNA analysis; ii) the use of negative controls; iii) the reproducibility of the results and independent replication; iv) cloning and sequencing the PCR products; v) testing the molecular behavior; vi) quantitation; vii) phylogenetic sense.

In case of NGS reads, one can use computational methods to identify characteristic patterns of aDNA damage to evaluate the authenticity of results, making the replication criteria obsolete (Linderholm, 2016). However, DNA contamination remains a critical concern in aDNA studies, primarily due to the limited sensitivity of computational methods and to the possibility of cross-contamination between samples (Llamas et al., 2017).

3. MtDNA as a genetic system in the research of human population history

Since the emergence of aDNA research, the preference for a certain genomic region has also been driven by the availability and progress of technology required to analyze and interpret genetic data. The most frequently used genetic system to assess the molecular diversity of past human populations has been the mitochondrial DNA, due to a combination of peculiar properties and practical concerns (Galtier et al., 2009).

3.1. Properties

Mitochondria, cytoplasmic organelles that generate cellular energy, have a small genome, independent (autonomously replicating) of the nuclear DNA (Schatz et al., 1964). As opposed to nuclear DNA, the mitochondrial chromosome is organized as a circular double-stranded molecule that shows substantial similarity to bacterial genomes. The complete sequence of the human mitochondrial genome is 16,569 bp in length and contains 37 genes, involved in cellular respiration, the main function of the mitochondria (Anderson et al., 1981). Besides the coding region, mtDNA includes a fragment of about 1,100 bp termed ‘control region’ because it has mainly regulatory functions. Human mtDNA has been extensively used as a molecular marker to study human evolution, migration and population histories due to its particular features that

make it suitable for such studies: high copy number, maternal inheritance, lack of recombination, and fast mutation rate (Kivisild, 2015).

3.2. MtDNA sequence variation in human populations

The history of mutational events can be reconstructed and visualized using phylogenetic trees that reveal the evolutionary relationships between DNA sequences of a population or a species. Related mtDNA lineages which share a particular set of polymorphisms (diagnostic mutations) are assembled into 'haplogroups' that have evolved from the same ancestor, and thus represent major branch points on the mtDNA phylogenetic tree. The most common branches of the mtDNA phylogenetic tree (haplogroups) were labeled with letters of the Latin alphabet (Torroni et al., 1993). The first mtDNA haplogroups were labeled with the A, B, and C letters and were assigned to genetic variation in Native Americans (Torroni et al., 1993). The letters H-K are associated with European mtDNA lineages (Torroni et al., 1994), whereas the oldest mtDNA haplogroup found in Africa is named L (Chen et al., 1995). The rapid accumulation of whole mtDNA sequences from worldwide populations led to the construction and subsequent updates of the human mtDNA phylogenetic tree (van Oven and Kayser, 2009). The currently used mtDNA tree (mtDNA tree Build 17: http://www.phylotree.org/) has a robust structure and gives a comprehensive view of the human genetic evolution from a matrilineal perspective (van Oven, 2015).

The sequence of the human mtDNA genome, the Cambridge Reference Sequence (CRS) was generated in 1981 and it has since been used as a reference sequence to record the human mtDNA polymorphisms (Anderson et al., 1981). Eight years later it was replaced by an updated version (*r*evised *C*ambridge *R*eference *S*equence, rCRS) in which the initial sequencing errors were eliminated (Andrews et al., 1999). It is now known that the reference sequence belongs to the recently coalescing European haplogroup H2a2a1 (van Oven and Kayser, 2009).

3.3. Methods for typing mtDNA

3.3.1. Amplification and sequencing of HVS by Sanger method

The traditional approach is based on target selection and amplification by PCR. The genetic fragments of interest are usually amplified from the DNA extract by using several short and overlapping target fragments (< 200 bp) required due to the degraded nature of aDNA molecules (Gabriel et al., 2001). The most commonly targeted region is the D-loop (mainly HVS-I) of the human mitochondrial genome as it is the most polymorphic and it contains phylogenetic relevant information. The primary method used for sequencing is the widely used Sanger method, now called the first generation sequencing technology.

3.3.2. Next Generation Sequencing of the complete genome

Although PCR-based strategies are presently the most effective in targeting DNA regions of interest, the NGS has been increasingly gaining interest for several reasons (Vai et al., 2016). Sanger sequencing has a low throughput and as a consequence is expensive for sequencing vast regions, whereas NGS platforms allow data output from billions of DNA fragments in a single sequencing run, and therefore the cost is substantially reduced (Rizzi et al., 2012). Then, the steps required to prepare the samples for NGS sequencing are less time-consuming. Another advantage of NGS is the possibility to recover the genetic information stored in short DNA molecules (30 bp) which are abundant in ancient degraded samples and not detectable by PCR (Dabney et al., 2013a). Moreover, because the ends of the original molecules are not modified, using the appropriate bioinformatics tool one can evaluate the damage patterns to discriminate between the authentic sequences and the exogenous ones (Vai et al., 2016). These aspects led to a substantial increase in sequence reads which created a need for novel algorithms and computational resources to process, store, and spread the sequence data (Muir et al., 2016).

Currently, there are four frequently used platforms for massively parallel sequencing that rely on more or less similar library preparation, sequencing chemistry and throughputs (Knapp and Hofreiter, 2010), but the most widely used in aDNA research field is the Solexa system, marketed by Illumina, essentially because the read length (75 or 100 bp) is suitable for short fragments as are the ancient ones (Metzker, 2010). There are three key steps for generating sequence reads: (i) library preparation; (ii) target enrichment; (iii) NGS sequencing.

4. The history of Europe revealed by aDNA research

4.1. Genetic connections in the European Bronze Age

The Eurasian Bronze Age (3,000-1,000 BC) was a very dynamic period involving large-scale population migrations, replacements, and admixtures that dramatically marked the genetic landscape of modern European and Asian human populations (Allentoft et al., 2015).

Large-scale studies (Allentoft et al., 2015; Haak et al., 2015) based on genomic data of ancient Yamnaya herders from the region of the Pontic-Caspian steppe revealed massive migration into Europe's heartland during the Early Bronze Age. The steppe populations were a mixture of at least two elements: descendants of Eastern European hunter-gatherers of the Mesolithic on one side, and of ancient Caucasus hunter-gatherers on the other side (de Barros Damgaard et al., 2018). The massive migration of steppe herders into Central and Northern Europe and their admixture with local Neolithic people lead to the formation of the Corded Ware culture. Another eastward expansion was confirmed by the great genetic similarities between Yamnaya and the Early Bronze Age Afansievo culture in the Altai-Sayan region (Allentoft et al.,

2015). These waves of migration might have been triggered by technological innovations associated with horseback riding, for example, as seen in the Yamnaya culture (Nielsen et al., 2017).

The complex aDNA study on the first farmers of southeastern Europe showed that this geographical region "served as a genetic contact zone between east and west over thousands of years", with sporadic genetic links to the steppe populations which occurred up to 2,000 years earlier than the arrival of steppe genetic signatures in the Central and northern Europe (Mathieson et al., 2018). Even though the genomic history of different European regions was illustrated, primarily from the Mesolithic to the Bronze Age, the pattern of these genetic connections remains to be verified by particular facts relating to each situation.

4.2. Archaeogenetics of the European populations in the Middle Ages

In comparison to other historical transects, there is a paucity of archaeogenetic data available for medieval European populations, in particular for the southeastern region. Currently, the genetic landscape of this age appears as a puzzle of dispersed, small, known pieces. Until recently, most of the aDNA studies on the medieval period reported only the control region of the mitochondrial genome plus some informative coding region SNPs for a small number of samples (about 10-30) per analyzed population: proto-Bulgarians (Nesheva et al., 2015); Cumanians (Bogacsi-Szabo et al., 2005); Lombards (Alt et al., 2014; Vai et al., 2015); Slavs (Csakyova et al., 2016; Juras et al., 2014), Vikings (Krzewinska et al., 2015); Spaniards (Alzualde et al., 2006). More in-depth ancient population genetic investigations were performed on medieval samples recovered from the current territory of Hungary (Csosz et al., 2016; Neparaczki et al., 2017; Tomory et al., 2007). By far, the most comprehensive study for this region and historical period is the one published by Neparaczki et al. (2018). By sequencing and analyzing about 100 complete mitochondrial genomes from early Conquerors, the authors show that the Conquerors most probably descended from steppe nomadic people and they did not have a major contribution to the gene pool of the Carpathian Basin (Neparaczki et al., 2018). Recent interdisciplinary studies on barbarian migration provided new insights on the social organization and migratory patterns associated with the Longobards, barbarian people, originally from Pannonia, that invaded Italy in the 6th century (Amorim et al., 2018; Vai et al., 2018). Even though ancient genomic research pinpointed some of the puzzle pieces on medieval Europe's archaeogenetic map, many blank spots remain to be filled.

4.3. Modern and ancient genomes from Romania

Ancient DNA studies using archaeological material from the current Romanian territory are scarce and being mostly restricted to distinct past periods such as the Early Neolithic, the

Late Bronze Age (Hervella et al., 2015). Probably the most outstanding finding is a 37,000-42,000-year-old human fossil from Peștera cu Oase (Trinkaus et al., 2003). The genetic analysis of this specimen provided valuable information regarding the relationships of modern humans and Neanderthals. It was revealed that 6-9% of the genome of the Oase individuals is derived from Neanderthals, more than any other modern human sequenced to date (Fu et al., 2015). The whole mitochondrial genome of a 35,000-year-old *Homo sapiens* from Peștera Muierii was identified as a basal haplogroup U6*, absent from previously analyzed ancient or modern genomes, which supports the hypothesis of a back-migration to Africa from Eurasia at beginning of the Upper Palaeolithic (Hervella et al., 2016). Genome-wide data of 12 samples from Romania, dated from Mesolithic to Neolithic, has recently become available in a study aimed to clarify the genetic history of southeastern Europe (Mathieson et al., 2018). Four other genomes from Iron Gates prehistoric samples were reconstructed and their analysis showed that the Neolithization process in the Lower Danube basin relied on the movement of both people and ideas (Gonzalez-Fortes et al., 2017).

Currently, most information on the genetic landscape of Romanian populations is inferred from mtDNA diversity in present-day inhabitants as reflected by the analysis of the mitochondrial control region, using the classical approach. A few studies attempted to evaluate their genetic relationship with other Eurasians (Hervella et al., 2014; Richards et al., 2000; Turchi et al., 2016), whereas some focused on mtDNA variation in ethnic minority groups (Bosch et al., 2006; Brandstatter et al., 2007; Mendizabal et al., 2012). It has been reported that the pattern of mtDNA diversity in about 400 Romanians is rather geographically homogenous across the country, the genetic pool is mainly composed of West Eurasian lineages (Turchi et al., 2016). Another study showed that there is some degree of genetic differentiation between the Romanian groups living within the Carpathian basin (North Romania) and those from outside the Carpathian range (South Romania) (Hervella et al., 2014). To shed light on possible past migratory routes, another study pointed that contemporary distribution pattern of mtDNA haplogroups in the historical provinces (Wallachia, Moldavia, and Dobrudja) of Romania is mostly governed by genetic affinities towards the Balkans, whereas the Transylvanian population is more closely related to Central European groups (Cocos et al., 2017).

II. Aims of the study

The first aim of this study was to provide genetic evidence for local historical populations from southeastern Romania (Dobruja), a geographical region that served over time as a passageway for migrations at the crossroads between East and West. The sampling was focused on the territory of Dobruja and two opportune archaeological sites were considered; the medieval necropolis of the Capidava fortress and the Bronze Age tumulus from Mireasa.

For each of these sites, the main purpose was to determine the genetic architecture of maternal lineages which can reveal the genetic diversity, kinship, residence rules, as well as family and population structure, all insights into the local genetic picture. The second objective was to expand this image by comparing the mitochondrial diversity of the sampled populations to other published populations, dated to the same historical periods. The genetic affinities among them can provide a better understanding of past population movements and interactions. The third objective was to draw a genetic comparison from a mitochondrial perspective between populations from the same geographical area but dated to different periods (Bronze Age, medieval, and modern) in order to detect the genetic changes over time in southeastern Romania. Another objective, common for individuals from both archaeological sites, was to make use of increased resolution of the full-length mitochondrial genomes in a phylogeographic approach in order to trace the possible genetic origins of the sampled individuals. Given the particular features of Capidava necropolis, in which two types of burial practices were identified (single Christian graves and multiple burial), it seemed appropriate to evaluate the genetic relationships between the two groups of individuals buried in contrasting fashions, in the same necropolis.

The second aim of this study emerged as a consequence of the methodological requirements of the aDNA research field. The available protocols for aDNA and protein analysis of ancient bones and teeth involve repeated damaging samplings of a specimen, thus increasing the extent of unwanted loss of valuable archaeological material. Therefore, we aimed to develop a combined method for aDNA and protein isolation from archaeological samples to minimize the damage to valuable material and to make use of the information stored in both ancient biomolecules.

III. Materials and methods

1. Archaeological sites and sample information

The collections of human skeletal remains (29 individuals) processed and discussed in this study were retrieved from two major archaeological sites (Capidava and Mireasa) from the current southeastern territory of Romania, Dobruja region.

2. Physical anthropology analysis of the archaeological remains

The anthropological investigation was carried out to gather information such as age at death, sex, traces of injuries, pathological conditions, and stress markers that can assess the demographic structure and epidemiological conditions of this group of ancient individuals. The availability of dental specimens with good preservation status was a cutoff selection criterion for subsequent molecular genetic investigations of samples.

2.1. Assessment of biological profile

The osteological examination was performed using previously described standard guidelines (Buikstra and Ubelaker, 1994; Steckel et al., 2011).

2.2. Pathological conditions and traumatic injuries

Each skeleton was assessed with regard to dental and skeletal pathological features. The former included *antemortem* tooth loss, dental abscesses, dental calculus and periodontitis, carious lesions, and dental enamel hypoplasia (DEH) (Buikstra and Ubelaker, 1994; Steckel et al., 2011). During the initial assessment and for the differential diagnosis we followed the guidelines (Aufderheide and Rodriguez-Martin, 1998; Ortner, 2003; Roberts and Manchester, 2005; Waldron, 2009). Trauma and fractures were recorded following the methods described by Lovell Nancy (1998) and Buikstra and Ubelaker (1994).

3. Molecular analysis of the ancient DNA

3.1. Traditional approach for reconstruction of the mitochondrial HVS

The most polymorphic region of the human mitochondrial genome, the control region was analyzed using the classical approach which is based on target selection and amplification by PCR.

3.1.1. Sample preparation

The biological material used for DNA isolation was represented by dental samples for each tested individual. After the external surface of the dental samples was decontaminated, powdered tissue, required for aDNA extraction, was obtained using a dental micro-drill.

3.1.2. Standard aDNA extraction

The DNA extraction process was performed following either of two routinely applied protocols. The first protocol (P1), used for the study of the medieval Christian Capidava samples, is a silica-based spin column method described by Yang and his collaborators (Yang et al., 1998). The second protocol (P2) was used for the analysis of the Bronze Age samples from the Mireasa barrow. For each sample, DNA was isolated following a published silica-based protocol, specially designed to target ultra-short molecules (Dabney et al., 2013a).

3.1.3. Dual DNA-protein extraction

This section is part of the published article: *Rusu I, Paica I, Vulpoi A, Radu C, Mircea C, Dobrinescu C, Bodolica V, Kelemen B (2018) Dual DNA-protein extraction from human archaeological remains. Archaeol Anthropol Sci https://doi.org/10.1007/s12520-018-0760-1*.

3.1.4. Amplification of hypervariable segments (HVS)

The amplification of both hypervariable segments (HVS-I and HVS-II) of the human mitochondrial genome was performed using an amplification strategy designed for highly degraded samples (Gabriel et al., 2001). Additionally, several phylogenetically significant coding region SNPs of the mitochondrial genome were amplified when the haplogroup assignment based on the genetic profile of the HVS was ambiguous.

3.1.5. Cloning and sequencing of aDNA

When no contamination was detected, the amplification products were cloned using the Sticky-End Cloning Protocol available with the CloneJet PCR Cloning Kit (Thermo Scientific, Waltham, USA). The recombinant plasmids were sequenced at Macrogen Europe (Amsterdam, The Netherlands) using the standard primer pJET1.2R. All resulting sequences were aligned using the ClustalW algorithm for multiple sequences included in BioEdit Sequence Alignment Editor v. 7.2.5.0 (Hall, 1999). The sequence polymorphisms in mtDNA were compared with the revised Cambridge Reference Sequence (rCRS, NC_012920) (Andrews et al., 1999), and then the automated alignment was manually checked. Haplogroup determination was carried out according to the mtDNA phylogeny of PhyloTree build 17 using the HaploGrep2 web application (van Oven, 2015; Weissensteiner et al., 2016).

3.1.6. Authentication criteria

During all experimental steps, standard guidelines for the analysis of ancient samples were followed (Gilbert et al., 2005; Paabo et al., 2004; Poinar, 2003) and several precautionary measures were undertaken to prevent and identify contamination by modern DNA. Two subsets of samples were independently processed by two separate research groups.

3.2. Next-Generation Sequencing

3.2.1. DNA extraction and library preparation

The undertaken experimental steps were previously described in detail by Modi et al. (2017) and Tassi et al. (2017). For each sample, DNA was extracted following an improved silica-based technique that allows for the recovery of molecules as short as 50 bp, prevalent in ancient samples (Dabney et al., 2013a). Double-stranded Illumina libraries for high-throughput sequencing of damaged DNA were constructed using a custom protocol (Meyer and Kircher, 2010) that consists of distinct phases of blunt-end repair of the ends of molecules, adapter ligation, and an indexing PCR required to add barcodes to each sample.

3.2.2. Mitochondrial DNA capture and sequencing

Custom made long-range PCR products were used as probes for hybridization capture of complete human mitochondrial genomes from the indexed libraries in a bead-capture method described in Maricic et al. (2010).

3.2.3. Bioinformatics analysis

Paired-end Illumina reads were processed using the pipeline described by Modi et al. (2017). Mitochondrial haplotypes for each sample were determined with the use of HaploGrep (Weissensteiner et al., 2016), based on PhyloTree (van Oven, 2015) build 17.

4. Comparative analyses using published mtDNA data

The mitochondrial data generated in this study were discussed and compared to the information available in the published literature in order to identify the genetic connections between the sampled individuals/populations and other ancient and modern representatives which could subsequently provide traces of evidence concerning past migratory routes and origin of the analyzed populations.

4.1. Additional and comparative ancient and modern mtDNA data

The mtDNA data retrieved from literature was arranged into four datasets: 495 medieval HVS-I sequences; 15368 modern Eurasian HVS-I sequences; 387 complete mtDNA sequences that match the haplotypes of the analyzed samples, 282 ancient and modern complete mtDNA sequences.

4.1.1. Mitochondrial DNA population genetic analyses

Principal Component Analysis (PCA) was performed based on mtDNA haplogroup frequencies and executed using the *prcomp* function of the built-in R stats package, R version 3.3.2 (R Development Core Team, 2016). The hierarchical clustering analysis was performed on all PCs using Ward's agglomerative method (Ward, 1963) and Euclidean distance measurement

and was calculated by means of *pvclust* function (Suzuki and Shimodaira, 2015). The significance of each cluster is given as an approximately unbiased (AU) p-value, as a percentage, and it was calculated by multi-scale bootstrap resampling with 10,000 replicates.

Pairwise population differentiations (F_{ST}) were computed from haplotypes of uniform sequence length in Arlequin 3.5.2.2 software (Excoffier and Lischer, 2010). The most appropriate nucleotide substitution model and the estimate of gamma parameter were determined with jModeltest 2.1.10. (Darriba et al., 2012; Guindon and Gascuel, 2003). The pairwise F_{ST} values were calculated assuming a Tamura & Nei substitution model (Tamura and Nei, 1993), 10000 permutations and an allowed level of missing data of 0.05.

Shared haplotype analysis (SHA) was performed by counting the absolute and relative shared haplotypes. The results of each type of analysis were visualized in a two-dimensional space using the appropriate libraries of R 3.3.2 (R Development Core Team, 2016).

4.1.2. Phylogenetic analysis

Median-Joining networks were built in PopART (Leigh and Bryant, 2015) with default settings. The phylogeographic relations were inferred based on the geographic origin of the closest similar sequences from literature.

IV. Results and discussion

1. Maternal ancestry of Christians and inconsiderately buried individuals from medieval Capidava necropolis

Within the historical province of Dobruja (southeastern Europe), one can find tracks marking the passage of multiple populations (*e.g.* Greeks, Romans, Byzantines, Pechenegs, Tatars, etc.) (Barnea and Ștefănescu, 1971). A witness of regional changes is the Capidava fortress and its associated settlement, from the beginning of the 2nd century AD when the Romans settled and became aware of its strategic importance, until the 11th century when the Pecheneg invasion ended the Byzantine inhabitation of this location (Florescu, 1946). Capidava acts, from this perspective, as a gate of access for migratory populations towards Western Europe and also as a trade center with an extremely complex history during the Middle Ages. Through the 10th century AD when Capidava is alternatively dominated, in the context of continuous conflicts between the Byzantine Empire, the Kievan Rus' and the proto-Bulgarians, by each of them. Questions regarding the impact and extent of these past political and associated demographic events, that left imprints on the local genetic structure and influenced the current European gene pool, remain unanswered, even though, the first elements of evidence could be inferred from the archaeological context (Pinter et al., 2011).

1.1. Archaeological background

Two sets of individuals discovered in the same archaeological site (Capidava), but with distinct archaeological backgrounds were genetically analyzed: the B terrace Christian individuals and the C58-multiple burial pit from Information Center area.

The osteological remains belonging to the 15 individuals discovered in the B terrace displayed a similar burial pattern: the bodies were usually individually inhumed in simple quadrilateral graves in a supine position with the hands assigned either on the pelvis or on the chest, having an East-West orientation (Pinter et al., 2011). The position of the skeletons along with the presence of few associated bronze artifacts (two button pendants and a hoop earring) are indicators for a Christian burial custom of the 10th-11th century AD.

In contrast to the burial custom observed for the B terrace individuals, the lack of care for the dead is conspicuous in case of the 10 individuals of the C58 archaeological complex. The skeletons were found on the bottom of an elliptical pit, with no funeral inventory present. They showed no clear pattern of organization which suggests that they were rather disposed of into the pit, as opposed to ritual deposition, possible due to haste and/or lack of consideration for the dead. The disorganized body positions and the absence of religious rites specific to the medieval period observed in the C58 complex suggest that these individuals were perhaps outsiders

(geographical, social or religious), unwanted or without rights in the local community whose representatives are not evident at this point in the adjacent necropolis.

1.2. Biological profile, pathology, and trauma

The skeletal material recovered from C58 archaeological complex is composed of seven adult and three subadult individuals. Sex distribution is rather imbalanced, as four of the individuals displayed male-typical morphological features (Cap-C58-1, Cap-C58-2, Cap-C58-3, and Cap-C58-5) and the remaining two exhibited skeletal markers which indicate that they are probably females (Cap-C58-6, and Cap-C58-11). The pathological conditions identified on these human remains (dental enamel hypoplasia, *cribra orbitalia*, porotic hyperostosis, dental disease, periosteal inflammation, and osteolytic lesions on the vertebrae) possibly reflect the poor life quality of this group of individuals. When comparing the presence of pathological features between the individuals from the C58 complex and the ones from the Christian necropolis, a slight increase in the incidence of stress indicators can be seen. The male individuals recovered from the C58 funerary assemblage exhibited signs of violent traumas.The pattern of physical trauma observed here indicates a violent death for nearly half of the individuals, all of them males, which might be the result of some kind of skirmish.

The osteological analysis of the individuals recovered from the B terrace showed that among the 11 individuals considered for molecular analysis six were subadults, 3 were females and the remaining two were males. The age category within this group varies from 38-40 prenatal weeks (Cap-M6) to 39.2-56 years (Cap-M3). In contrast to the C58 group (where all males displayed traumatic injuries), these individuals do not show signs of a violent death.

1.3. Genetic variation of the medieval Christian population reflected by the mtDNA control region

This section is part of the published article: *Rusu I, Modi A, Vai S, Pilli E, Mircea C, Radu C, Urduzia C, Pinter ZK, Bodolica V, Dobrinescu C, Hervella M, Popescu O, Lari M, Caramelli D, Kelemen B (2018) Maternal DNA lineages at the gate of Europe in the 10th century AD. PLOS One 13:e0193578.*

1.3.1. Authentication of the results

The mitochondrial control region of ten medieval Christian individuals was successfully amplified and typed using a variety of techniques in three different laboratories. All sequences that were replicated, along with the typed coding region positions, negative control results and the presence of characteristic *post-mortem* DNA damage in all cloned mtDNA inserts suggest the generated sequences are authentic. Inconsistent results across laboratories were obtained only for Cap-M5 individual and therefore it was excluded from all downstream analyses.

The results from both extraction methods (silica-columns and dual DNA-protein) were equivalent for medieval or more recent samples. Therefore, it is worth emphasizing that the proposed protocol can be used to obtain authentic genetic information.

1.3.2. Mitochondrial hg composition and genetic relationships among individuals

The 10 consensus sequences that were successfully reconstructed for the medieval Christian Capidava individuals, spanning positions 37-357 and 16009-16390 belong to 8 haplotypes (haplotype diversity of 0.956). Identical haplotypes were identified in two groups of two individuals originating from neighboring graves (Cap-M3 and Cap-M4 belong to hg R0a2'3; Cap-M9 and Cap-M11 could be classified into N9a). The high number of intra-cemetery maternal relations relative to the population size suggests that family connections were significant considerations of spatial burial distribution. In order to avoid overrepresentation of mtDNA lineages due to potential family relationships, duplicate sequences were removed from the subsequent statistical analysis. The medieval Christian Capidava population analyzed here has a predominantly Western Eurasian haplogroup composition (H, R0, U3, U5, and V), the Eastern Eurasian component being represented by hg N9a with a frequency of 12.5%.

1.3.3. Genetic connections to other medieval populations

The PCA of the 15 medieval populations, computed based on mtDNA haplogroup frequencies, shows a rough clustering between the investigated population and the Cumanians from Hungary along the PC1 and PC2 components (34.82 % of the variance is displayed) (Fig. 1). This assembly is fairly distantly positioned with respect to the remaining medieval European populations, being displayed at the opposite pole on PC2 from the Avars and the Hungarian conquerors, even though they are geographically close. Close genetic ties are shown between populations derived from the same geographical areas, but this pattern is observed only for the medieval populations from Southern Europe and those from Northern Europe. Such connections were also previously noted by Csakyova et al. (2016).

Hierarchical clustering shows a very similar diagram to the PCA and reveals the clustering of the medieval Romanian population with the Cumanians from Hungary, a fact which can be explained by the interplay of all PCs in this analysis.

The inter-population genetic distances were calculated based on control region haplotypes of uniform sequence length, ranging from nucleotide position 16050 to 16383. When compared to other populations dating from similar historical periods, the medieval population from the current Southeastern territory of Romania showed non-significant differences from all populations ($p>0.05$), the smallest p-values, associated to the highest F_{ST} values, being observed in medieval population from Slovakia (SVK-med) with $p = 0.08336\pm0.0028$ and $F_{ST} = 0.05047$

and in medieval Italians (ITA-med) with p = 0.12999±0.0036 and F_{ST} = 0.04055. The smallest genetic distances were observed in medieval Bulgarians (F_{ST} = -0.02839), Danish Vikings (F_{ST} = -0.02839), Hungarian Avars (F_{ST} = -0.0207) and Cumanians (F_{ST} = -0.0207).

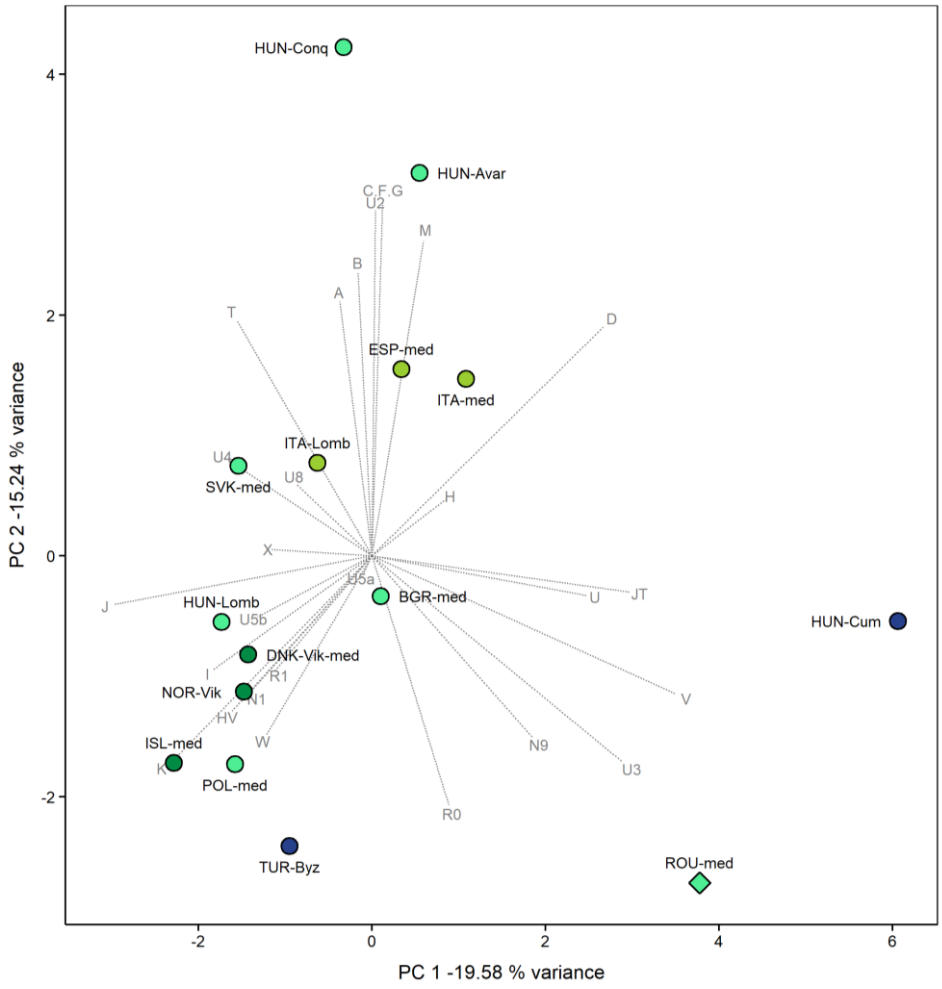

Fig. 1. PCA plot of the first two components (34.82 % of variance) of medieval populations. ROU-med includes 8 Christian individuals from medieval Capidava.

The SHA performed on the medieval populations reveals a distinct result from the F_{ST} calculation, showing that the medieval population from Romania shares most of the haplotypes with medieval populations from Spain and Italy. This high proportion of lineage sharing between these populations, probably occurred due to the presence of the high percentage of the most widely distributed mtDNA haplotypes, H lineages, in modern populations of Eastern Eurasia, being therefore phylogeographically uninformative in this instance due to small data set and low HVS-I resolution.

1.3.4. Genetic links to modern populations

The PCA plot of the contemporary Eurasian populations and the Capidava population shows the agglomeration of the European ones on the PC1 (23.38% variance) and PC2 (15.73%

variance) components, in contrast to modern Asian populations which are scattered (Fig. 2). Along PC1, the medieval Romanian population is more distant from the modern European groups than the Asiatic ones, but the contribution of the second PC shows that its connections to the latter populations are not so tight. Of the European populations, those that seem to have a higher genetic affinity with the one analyzed in this study, are mainly the Slavic populations from Eastern Europe, as well as those originating in Northern Europe.

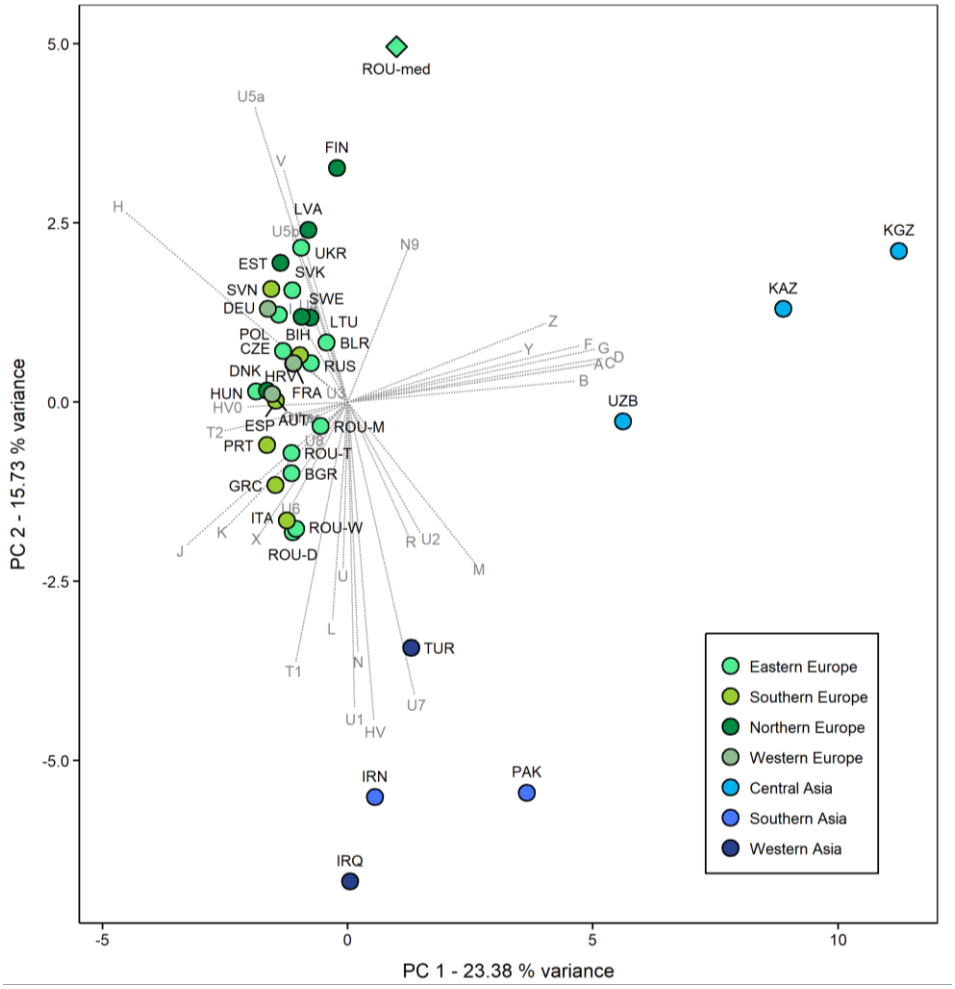

Fig. 2. PCA of the investigated medieval and present-day populations showing PC1 and PC2. ROU-med includes 8 Christian individuals from medieval Capidava.

The MDS plot illustrates a similar pattern to the one reflected by the PCA, showing the aggregation of most of the modern European populations along the first two coordinates, whereas most of the Asian populations are disconnected. Of the Western Asian populations, the one from Turkey shows stronger affinities to the modern European ones and also to the Capidava population, a finding that can be somewhat explained by the prolonged interactions between the people from these regions (Turkic groups to which also the Cumanians and Pechenegs belong).

1.4. Complete mtDNA profile

The complete mitochondrial genomes were successfully reconstructed for 11 medieval individuals from Capidava necropolis, using hybridization capture in solution combined with high-throughput DNA sequencing.

1.4.1. Samples and bioinformatics analysis output

The resulted sequences reached the standard quality required to guaranty the reliability of the NGS data for all samples but Cap-M5 which was excluded from downstream analysis.

1.4.2. Refinement of haplogroup classification for Christian individuals

In addition to validating the mtDNA variants observed in the control region, the complete mitogenomic data of five Christian individuals allows for the refinement of haplogroup attribution that was originally made based on the mutational motifs of the hypervariable regions.

According to the high-resolution mitogenomic data, M2 sample belongs to V1a haplogroup, a typically Western Eurasian V sub-clade, currently dispersed, based on complete modern mitochondrial data across all over Europe and European Russia. NGS data ascertains the fact that M9 and M11 have indistinguishable haplotypes, and due to the presence of the T2287C transition, they can be further classified as N9a9. The genetic connection to Central Asia, already shown by the control region data, is now even more obvious as both M9 and M11 share their two private mutations (G228T and A15799G) with two modern individuals, one belonging to the Tubalar ethnic group (Altai Republic) and one belonging to the Kyrgyz ethnic group. The common H control region haplotypes of M15 and M17 can be also further narrowed. Sample M15 contains all defining mutations for H13a1a3, while the matrilineal variability observed in the complete sequence of M17 can be classified as H5e1a1 and shows the presence of private mutation A13731G. The further classification into V1a, H13a1a3, and H5e1a1 for the three Capidava samples points out to links much more geographically restricted to Central and Eastern Europe, thus reducing even more the strength of the apparent connection to medieval Italian, Spaniard and other peri-Mediterranean populations emerged from the analysis of the haplogroup H generic frequencies (SHA).

1.4.3. Mitochondrial haplogroup diversity of the multiple grave group

This section is part of the published article: *Rusu I, Modi A, Radu C, Mircea C, Vulpoi A, Dobrinescu C, Bodolică V, Potârniche T, Popescu O, Caramelli D, Kelemen B (2019) Mitochondrial ancestry of medieval individuals carelessly interred in a multiple burial from southeastern Romania. Sci Rep, 9, 961, doi:10.1038/s41598-018-37760-8.*

1.4.4. Distribution of maternal lineages and phylogenetic patterns for C58 samples

The increased level of resolution of full length mtDNA data allowed for the identification of direct links among closely related ancient and modern mitogenomes and for the precise assignment of geographic affinities. Phylogenetic analysis showed that three C58 haplogroups (H11a1, U4d2, and J1c15) are ubiquitous in Eurasia (Fig. 3), while the T2b clade is widely spread across Europe. The closest sequence matches elucidated using median-joining networks pointed to geographic affinities restricted to the Middle East in case of N1a3a and to the Mediterranean region for U6a1a1.

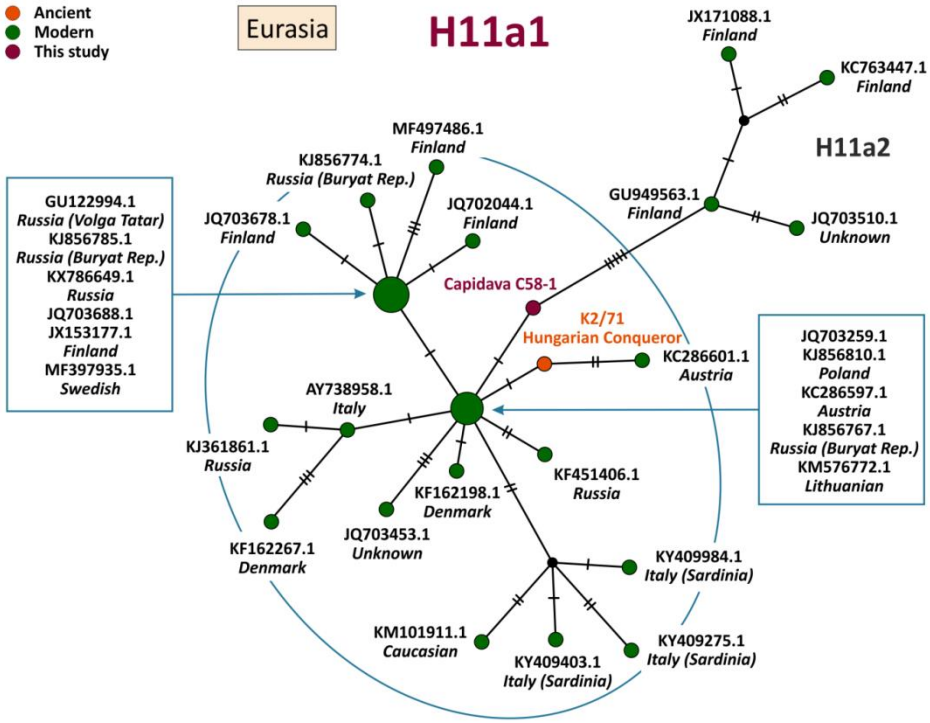

Fig. 3. H11a1 haplotype network.

1.4.5. Genetic relationships of medieval Capidava population with other ancient populations

The medieval population from southeastern Europe (all Capidava samples) showed the lowest distances from four ancient populations: Bronze Age population of the Balkan Peninsula (Balkans_BA) (F_{ST}= -0.0348), Srubnaya (SRU) (F_{ST}= -0.02367), Poltavka-Potapovka (PBA) (F_{ST}=-0.01677), and Hungarian Conquerors (HUN_Conq) (F_{ST}=-0.01325); these values were not significant ($p>0.05$). Five ancient groups (SBA, Scand, NEN, HUN_BA, and BBC) appeared most distant to the individuals in our dataset, displaying significant differentiation (Fig. 4).

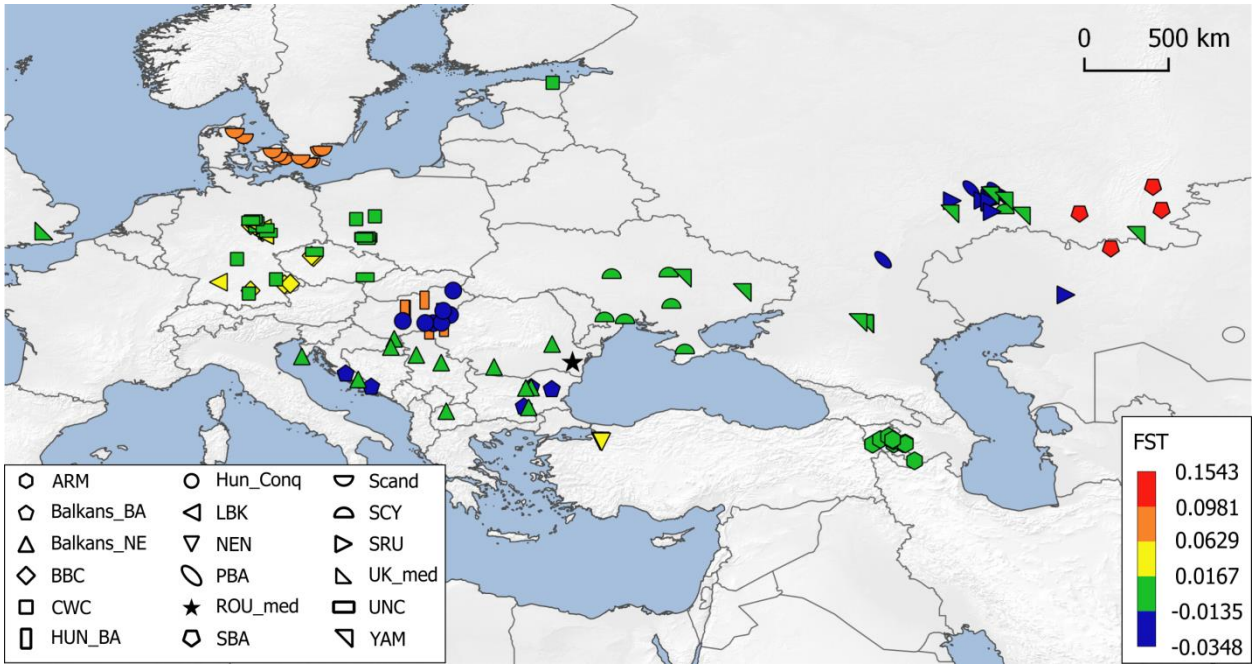

Fig. 4. Heatmap of F_{ST} and geographic distribution. Smaller pairwise F_{ST} values indicating less genetic distances are marked by blue shades. ROU-med includes 11 complete mitogenomes from Capidava. The map was created using QGIS 2.18.11 (QGIS Development Team, 2016.).

The MDS plot reflects the extremely tight connection between the ROU-med and Bronze Age population from the Pontic-Caspian steppe (Late Bronze Age Srubnaya culture and its ancestors Potapovka and Poltavka). The investigated medieval dataset also shows strong affinities to the Bronze Age population from the Balkans and to the Hungarian Conquerors, groups which are geographically close to the medieval population from Romania. Near Eastern Neolithic, Sintashta-Andronovo, and Bell Beaker seem to be the most distant populations. Therefore, the maternal genetic composition of the Capidava population as represented by all analyzed samples points to a mixed origin from multiple sources.

1.5. Genetic influences and possible origins of the individuals

The Christian Capidava medieval samples display a high haplotype diversity which may indicate a very dynamic genetic environment: intense local population turnover. Genetic duplicates in adjacent burial plots highlight possible familial affiliations, characteristic for Christian cemeteries. The presence of the Central Asian N9a haplotype in two Christian individuals seems to place Capidava in a genetic landscape dominated by Turkic influences, while common “European” lineages (H and V) point out to genetic connections much more restricted to Central and Eastern Europe.

The high diversity of mitochondrial haplogroups detected in the C58 archaeological complex (six individuals, six haplotypes) reflects the lack of close maternal relationships among individuals, and quite an intricate genetic history, as the identified subclades have diverse

geographic distribution (Eurasia, Middle East, Southern Europe). All the mtDNA information on the medieval Capidava population provides further evidence for genetic connections to the Hungarian Conquerors, a Bronze Age population from the Balkans and Bronze Age populations of steppe origin (Srubnaya and Potapovka-Poltavka).

2. Mitochondrial DNA lineages of Bronze Age and pre-modern individuals from Mireasa archaeological site

Two main assemblages (a large pre-modern necropolis and a small Bronze Age barrow) were discovered in 2012 near Mireasa village, located in Dobruja region. Archaeological samples dated to both historical periods were molecularly analyzed in order to to shed light on the genetic changes that occurred in this particular region.

2.1. Archaeological context and radiocarbon dating

The T38 barrow included 15 Bronze Age graves which might be associated to the Yamnaya culture based on the funerary context (entrance leading to a funerary room, wooden beams, crouched position of the skeletons, ochre powder). The scarce presence of funeral inventory seems to be a regional trait for Yamnaya culture (which distinguished the Yamnaya from the west of the Black Sea from their north pontic relatives). The other 147 archaeological complexes were presumably dated to the medieval period based on the funerary architecture, the position, and orientations of the skeletons, and analogies to other inhumation graves discovered in Dobrudja, but the direct radiocarbon dating of Mir-M66 showed that this individual is dated to the pre-modern period.

2.2. Osteological analysis

The morphological examination performed on the human remains of 12 Bronze Age individuals from Mireasa, shows that six of them are adults and the other six sub-adults. The sex could be determined for five specimens, all of them being males. The presence of dental enamel hypoplasia, *cribra orbitalia*, and porotic hyperostosis correlated with dental disease and periosteal inflammation, suggest that this population group experienced nutritional deficiencies and/or a possible low immunological status. However, the individuals were able to overcome these deficiencies, in all cases the lesions being healed.

2.3. Control region polymorphism pattern

Of the 12 Bronze Age individuals from Mireasa, nine samples were selected for molecular analysis, based on the preservation status of the teeth. The control region polymorphism pattern was obtained for other three pre-modern samples from Mireasa.

2.3.1. Most probable mtDNA haplogroup assignments for Bronze Age samples

Based on the variation observed at the mitochondrial control region level of Mireasa Bronze Age group, the haplogroup composition of this population seems to be restricted to few common Eurasian maternal lineages in equal percentages (H, K, HV, and U) and a non-H haplogroup (probably D which is particularly dominant in East Asia).

2.3.2. MtDNA haplogroups of pre-modern samples

The two HVS fragments were successfully reconstructed for three individuals from the pre-modern necropolis of Mireasa. The HVS consensus sequence of Mir-M47 has the sequence motifs for its classification into the basal East Eurasian haplogroup C that appears for the first time in the sample sets processed in this study. The haplogroup assignment for individual Mir-M67 is moderate because its sequences lack two diagnostic SNPs for haplogroup K (73G and 16311C). Their absence seems to be real as they were not present in either of the replicates. A derivative sublineage (HV13) of the HV haplogroup is present in the genetic pool of the pre-modern Mireasa samples (Mir-M69), but it is different from those identified in the Bronze Age samples proximately located. This particular haplotype was not frequently detected in historical populations. Currently, the only ancient specimen belonging to HV13 is a Late Iron Age Kangju individual from the Central steppe.

2.4. Complete mtDNA profile of Bronze Age individuals

The complete mitochondrial genomes were successfully reconstructed for two Bronze Age individuals from Mireasa T38 necropolis.

2.4.1. Bioinformatics analysis output

The resulted sequences reached the standard quality required to guaranty the reliability of the NGS data for both Bronze Age samples.

2.4.2. Phylogenetic and phylogeographic relationships

The complete mitochondrial genome retrieved for the two Bronze Age Mireasa individuals clarifies their haplogroup classification. The Mir-M160 sample contains all defining mutation motifs for U4a, a descendant subclade of the western Eurasian U4 haplogroup as well as a private mutation in the coding region, position 12172. This haplotype has a wide geographic distribution in modern European samples and seems to be well represented in ancient individuals as well (Fig. 5). The polymorphism distinguished in the complete mtDNA consensus sequence of Mir-M4 can be classified as KIc1, a derivative sub-branch of K1 (Fig. 6). The 498 deletion associated with this specific mitochondrial variant is absent, even though it appeared all clones generated using PCR coupled with Sanger sequencing. K1c1 is particularly common among

modern populations from northwestern Europe (Finnila et al., 2001; Raule et al., 2014) as well as in some Slavic countries (Behar et al., 2012; Malyarchuk et al., 2017).

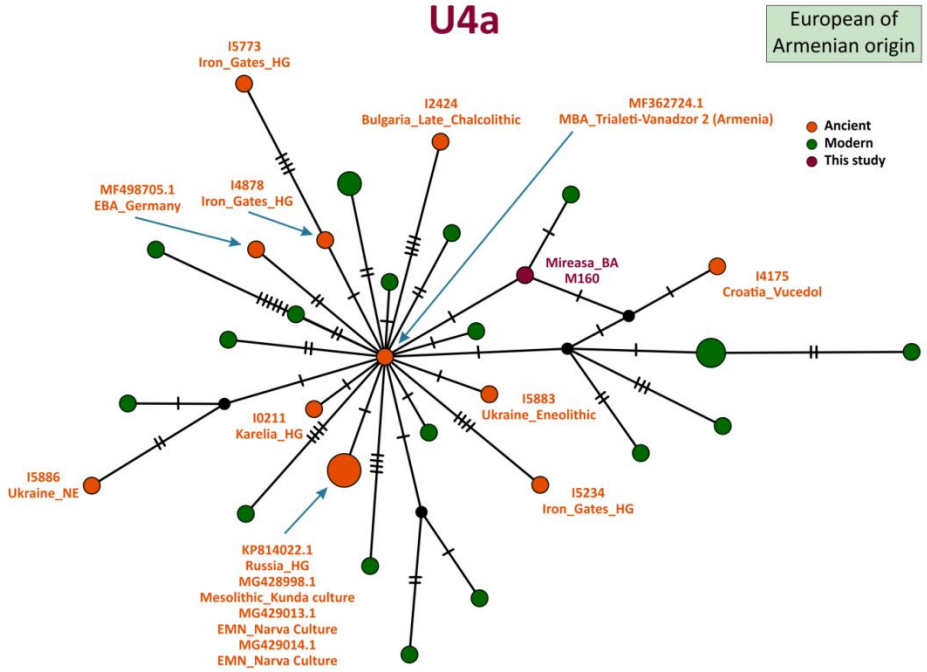

Fig. 5. U4a haplotype network.

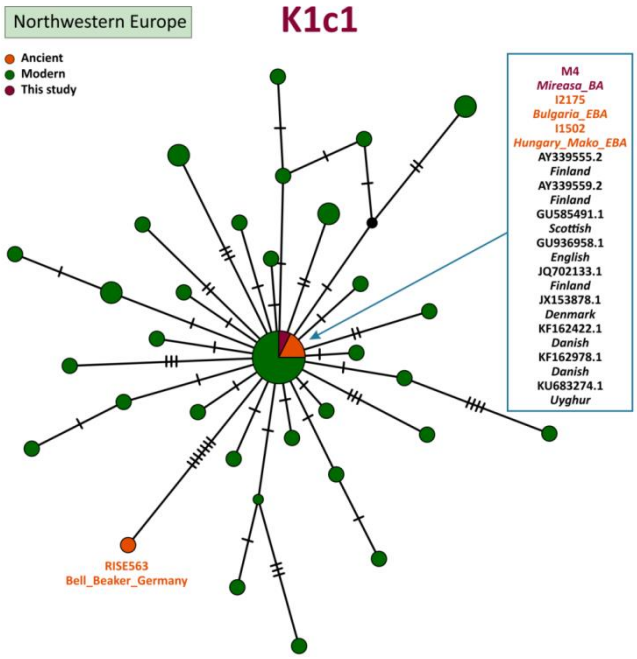

Fig. 6. K1c1 haplotype network.

V. Conclusion and prospects

This thesis provides new insights on the maternal population history from southeastern Romania, a geographic region that is scarcely represented on the European archaeogenetic map, but of great interest due to the complex social, cultural, and demographic past transformations. At the same time, a new protocol designed to co-purify DNA and proteins from archaeological human remains was developed in order to minimize the destruction of valuable biological material.

Currently, different geographic regions and historical periods are characterized by imbalanced datasets, be it due to the number of samples/region or due to differences in resolution of genetic targets, thus limiting the establishment of strong unequivocal analogies. Given our small data set, the main importance of the present study consists in supplying a list of mitochondrial variants for a region and a historical period completely lacking information, a situation often encountered for many other European medieval populations. In the age of NGS technology, a critical mass of data has to be attained in order to permit future phylogenetically, phylogeographically and demographically informative comparisons.

In the future, it would be ideal to implement the experimental protocols required for NGS in our lab in order to analyze ancient mitogenomes for regions of significant population flux with potentially many distinct migration or colonization events prior to modern times. Even though there is still room for sampling ancient mtDNAs, at some point, I would like to expand my research beyond this popular genetic marker and to perform genome wide-studies on archaeological specimens. The analysis of several independent loci would be extremely useful for inferring demographic history of populations, migration pattern, admixture, at a finer resolution.

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List of publications included in the thesis

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