Studia Antiqua et Archaeologica 22(2): 119–128

What can aDNA analysis tell us more on an old funerary discovery?

Neculai BOLOHAN[1], Florica MĂȚĂU[2],
Mitică CIORPAC[3], Dragoș Lucian GORGAN[4]

Abstract. *This study presents the first ancient mitochondrial DNA (amtDNA) results obtained by sampling human bones selected from an Early Bronze Age funerary context with the aim of identifying the haplogroup and starting to build an amtDNA reference database based on samples selected from Eastern Romania. The human bones analysed in this study were part of the Stoicani "Cetățuia" (Galați county) necropolis located in the Covurlui Plateau. The M6 funerary context does not contain any grave goods, his chronological and cultural characteristics being inferred based on its association with similar funerary contexts in the necropolis. The amtDNA obtained by analysing osteological remains attributed to the beginning of the Early Bronze Age in the Eastern Romania region helped us to identify the coexistence of different communities in the timespan characterized by accentuated human group mobility.*

Rezumat. *Acest studiu prezintă primele rezultate ADN mitocondrial vechi (amtDNA) obținute din analiza unor fragmente osteologice umane ce provin dintr-un context funerar atribuit Bronzului timpuriu, care au avut ca scop identificarea haplogrupului precum și începutul constituirii unei baze de date pentru ADN-ul mitocondrial vechi din estul României. Fragmentele osteologice provin de la un individ identificat în cadrul necropolei de la Stoicani "Cetățuia" (jud. Galați), care este localizată în Podișului Covurluiului. Contextul funerar analizat nu prezintă elemente de inventar asociate, atribuirea cronologică și culturală realizându-se pe baza asocierii cu celelalte morminte din necropolă. Analiza ADN-ului mitocondrial extras din fragmentele osteologice atribuite acestei secvențe de început a Bronzului timpuriu din estul României a permis identificarea coabitării diferitelor comunități umane într-o perioadă caracterizată printr-o mobilitate accentuată.*

Keywords: *ancient DNA, mitochondrial DNA, funerary context, Stoicani necropolis, haplogroups.*

[1] Faculty of History, "Alexandru Ioan Cuza" University of Iași; neculaibolohan@yahoo.com
[2] Interdisciplinary Research Department – Field Science, "Al. I. Cuza" University of Iași; florica.matau@uaic.ro
[3] Interdisciplinary Research Department – Field Science, "Alexandru Ioan Cuza" University of Iași; Faculty of Biology, "Alexandru Ioan Cuza" University of Iași; ciorpac.mitica@gmail.com
[4] Faculty of Biology, "Alexandru Ioan Cuza" University of Iași; lucian.gorgan@uaic.ro

Introduction

Until recently, much of what was considered to represent the backbone of the main research paradigms in archaeology came from the systematic survey and excavation of the archaeological sites followed by the anthropological investigations, based on which the patterns of the human migration and admixtures were inferred. The information obtained based on the physical features of the human remains was completed with the stratigraphic data and, when available, with the typological and stylistic analysis of the artefacts. Although important, these types of investigation often generated more questions than answers, and their result had a very limited value for the understanding of the cultural dynamics for different human groups in terms of acculturation or population movement.

Starting within the last two decades, the ancient DNA enquiries have been constantly modelling our fundamental understanding of the human prehistory and origin. The genetic analysis of ancient human remains can help us to assess some morphological features, such as the blood group, skin or eye colour and hair type. Direct access to DNA molecules sequenced from ancient human offers invaluable information about the biological characteristics and population dynamics of ancient communities.

Ancient DNA (aDNA) combined with archaeological and anthropological evidence provides a unique perspective on the interactions between communities, their organization, or subsistence strategies. The peculiarities of aDNA can make its study difficult, especially for human DNA. These facts can explain, among other factors, why there are relatively few paleogenetic studies on prehistoric populations from eastern Romania[5].

To enlarge the reconstruction of the history of the population dynamics from the Eastern Romania, within the *Genetic Evolution: New Evidences for the Study of Interconnected Structures. A Biomolecular Journey around the Carpathians from Ancient to Medieval Times* (GENESIS) project we have studied the genetic diversity of ancient communities, focusing, mainly, on the prehistoric ones, based on samples selected from old and more recent excavations which were made available to us by the museums and other institutions from the study area.

The main purpose of this study is to identify the haplogroup for the analysed osteological remains and, then, to trace the different patterns of mobility and coexistence of human groups from the beginning of the Early Bronze Age in the Eastern Romania region.

Archaeological context

The Stoicani area is situated in the contact zone between the hilly region of the Covurlui Plateau and the Prut river terraces, having more common features with the North Pontic

[5] BOLOHAN *et alii* 2016; GORGAN *et alii* 2016.

steppe region (Figure 1). Its strategic position made this region a natural crossroad for cultural, economic and social communication with the North Pontic steppe region during the Chalcolithic, Bronze and Early Iron Age.

The Stoicani "Cetățuia" necropolis contains 15 skeletons in flexed positions and painted with red ochre, grouped, probably, under a *tumulus*, which were attributed based on the associated artefacts to the *steppe communities* belonging to the so-called "transition period to the Bronze Age"[6] or, more recently, to the Early Bronze Age[7].

Although many aspects of the Early Bronze Age in Eastern Romania are still not very well understood, most of the researchers are in agreement with the coexistence of eastern elements (e.g. Jamnaja, Katakombnaja) together with some Chalcolithic/Early Bronze Age north-western influences (e.g. Globular Amphora, Trzciniec culture)[8].

The skeletal material under study comes from the old excavation directed by Mircea Petrescu-Dîmbovița between 1946–1949, when he identified the Chalcolithic settlement (Stoicani-Aldeni cultural group) and the Early Iron Age settlement[9]. In the present study we have sampled for the aDNA analysis the skeletal remains from M6 which does not contain any grave goods (Figure 2).

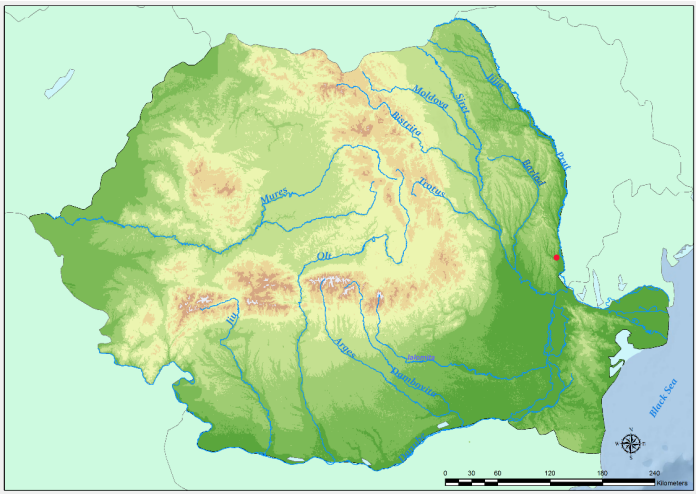

Figure 1. The location of the Stoicani "Cetățuia" necropolis

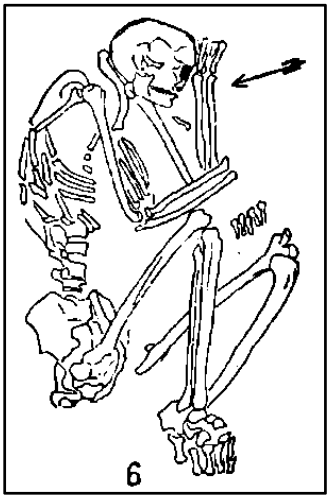

Figure 2. The M6 funerary context from the Stoicani "Cetățuia" necropolis (ap. Petrescu-Dîmbovița 1953)

[6] BURTĂNESCU 2002, 93–94.

[7] CHICIDEANU 2011, 224–285.

[8] BOROFFKA 2013, 4.

[9] PETRESCU-DÎMBOVIȚA 1953, 120.

The funerary remains from M6 were not consistently analysed in the literature. F. Burtănescu does not mention at all M6 among the funerary discoveries attributed to the Early Bronze Age[10]. When discussing the chronology of the Stoicani "Cetățuia" necropolis, he uses the chronological system suggested by P. Roman based on which the M6 grave belonged to a second phase of the Stoicani "Cetățuia" necropolis[11]. Although, he analysed, also, the funerary discoveries attributed to the transition period to the Early Bronze Age, he only mentions the Stoicani "Cetățuia" necropolis, without mentioning the specific graves[12]. In a more recent synthesis of the Bronze Age funerary customs, I. Motzoi-Chicideanu does not mention at all the Stoicani "Cetățuia" necropolis when he discusses the Early Bronze Age preliminary cultural sequences[13]; he only discusses the necropolis in the section dedicated to the *red ochre cultural phenomena* (Jamnaja and Katakombnaja cultures)[14], without discussing, specially, the M6 funerary context which is present only on the necropolis plan[15].

Material and methods

The analyzed sample is represented by one bone fragment (Figure 3) used for aDNA isolation and sequencing of hyper variable first part (HV1) within mitochondrial control region.

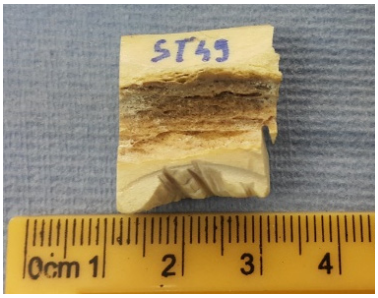

Figure 3. ST49 osteological sample — a fragment from the right femur.

To avoid external contamination during the procedures of aDNA isolation and amplification, the working has been exclusively used for ancient samples, being completely separated from the one where modern DNA is manipulated. UV irradiation of the working area was also used, as well as the sterile and RNA-se/DNA-se free laboratory materials and regents. Masks, gloves and labs coat were used during the samples manipulations. Amplification inhibitors may also be co-purified in the extracts obtained and its presence produces negative results.

The aDNA extraction from bone remains involved two main activities: samples preparation and extraction protocol. The bones were cleaned of debris and washed with distilled water, followed by a drying step. Further, the bones were washed with 10% NaOCl, dried and exposed to UV light for 15 minutes on each side. Finally, with a sterile bone drill a small area of the bone surface was removed and then bone powder was sampled, using a

[10] BURTĂNESCU 2002, 133.
[11] BURTĂNESCU 2002, 134.
[12] BURTĂNESCU 2002, 85.
[13] CHICIDEANU 2011, 190–213.
[14] CHICIDEANU 2011, 224–285.
[15] CHICIDEANU 2011, Plate 76.

reduced rotation speed, to avoid the temperature increase followed by an accelerate aDNA degradation. Every bone was prepared using the required standard protocols for aDNA analysis (facemask, gloves, and disposable lab coat), sterile and disposable tools in a controlled environment.

DNA isolation

For aDNA isolation, a DNA IQ (Promega, USA) commercial kit has been successfully used after a total demineralization step, using 40 mg of bone powder according to the manufacture protocol.

In order to identify any possible contamination that might have occurred in the different stages of the samples preparation and mainly in the aDNA isolation, two extraction blank controls and multiple PCR non-template controls were included in each amplification reaction. The rate of contamination for this analysis was less than 0.5%.

PCR and sequencing

The mitochondrial hyper variable region 1 (HV1) was amplified via PCR method using four pairs of primers (Table 1). The PCR was performed in a 25 µL reaction volume using GoTaq® Hot Start Polymerase (Promega, USA). The amplicons were purified using the Agencourt AMPure XP (Beckman Coulter, USA) and directly sequenced using the Genome Lab DTCS Quick Start Kit (Beckman Coulter, USA) in the CEQ 8000 Genetic Analysis System (Beckman Coulter). The sequence analysis was performed using the CEQ8000 instrument software. Multiple sequences have been analyzed and low frequency mutations were considered artefacts resulting from post-mortem aDNA damage.

Table 1. HV1 primers sets.

Locus	Pimers	Amplicon Size (bp)	Reference
MPS1A	F 5'-CCC AAA GCT AAG ATT CTA AT-3 R 5'-TAC TAC AGG TGG TCA AGT AT-3'	170	Gabriel *et al.*, 2001
MPS1B	F 5'-CAC CAT GAA TAT TGT ACG GT-3 R 5'-TGT GTG ATA GTT GAG GGT TG-3'	126	Gabriel *et al.*, 2001
MPS2A	F 5'-CCC CAT GCT TAC AAG CAA GT-3 R 5'-TGG CTT TAT GTA CTA TGT AC-3'	133	Gabriel *et al.*, 2001
MPS2B	F 5'-CAC TAG GAT ACC AAC AAA CC-3' R 5'-GAG GAT GGT GGT CAA GGG AC-3'	143	Gabriel *et al.*, 2001

Results and discussion

Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented[16]. Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown (Table 2). Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. Relative values of instantaneous r should be considered when evaluating them. For simplicity, the sum of r values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated. There were 381 positions in the final dataset. Evolutionary analyses were conducted in MEGA7[17].

Table 2. Maximum Likelihood fits of 24 different nucleotide substitution models.

Model	Parameters	BIC	AICc	lnL
HKY	41	1.772.597	1.488.572	-703.059
HKY+G	42	1.779.366	1.488.424	-701.974
HKY+I	42	1.779.563	1.488.621	-702.072
TN93	42	1.779.738	1.488.796	-702.160
TN93+G	43	1.786.696	1.488.838	-701.169
TN93+I	43	1.786.813	1.488.956	-701.228
HKY+G+I	43	1.788.471	1.490.613	-702.057
TN93+G+I	44	1.795.634	1.490.862	-701.169
GTR	45	1.804.419	1.492.732	-701.092
K2	38	1.805.682	1.542.409	-733.009

Table 3. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution.

Nucleotides	A	T	C	G
A	–	0.43	0.71	6.88
T	0.67	–	44.26	0.26
C	0.67	26.76	–	0.26
G	17.96	0.43	0.71	–

[16] NEI, KUMAR 2000.

[17] KUMAR *et alii* 2016.

Each entry shows the probability of substitution (r) from one base (row) to another base (column)[18]. For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies are 32.24% (A), 20.88% (T/U), 34.53% (C), and 12.35% (G) (Table 3). The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 381 positions in the final dataset.

The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Hasegawa-Kishino-Yano model[19] The tree with the highest log likelihood (-703.0586) is shown in Figure 4. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach,

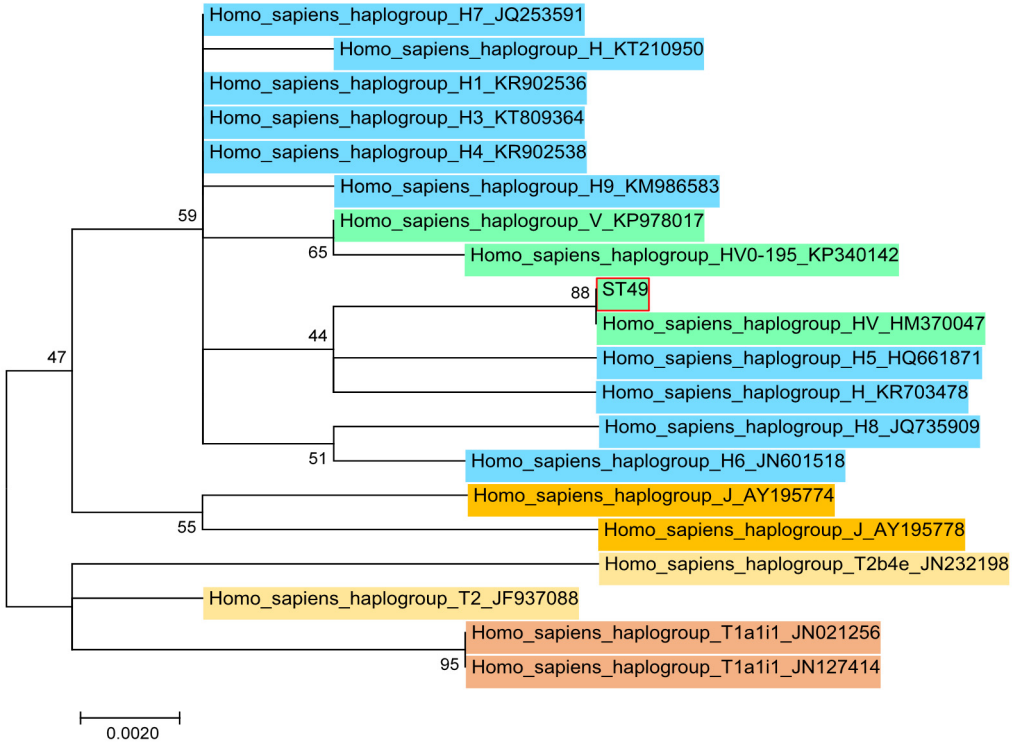

Figure 4. Molecular Phylogenetic analysis by Maximum Likelihood method
(the colour codes correspond to the Eupedia haplogroups colour charts)

[18] NEI, KUMAR 2000.

[19] HASEGAWA *et alii* 1985.

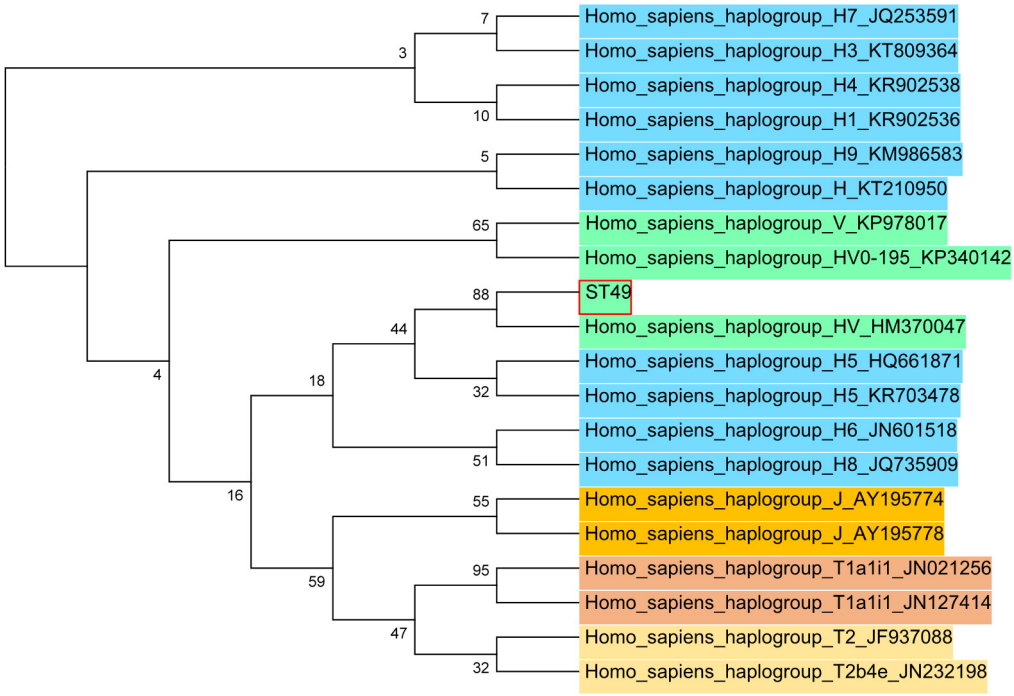

Figure 5. The ML bootstrap consensus tree
(the colour codes correspond to the Eupedia haplogroups colour charts)

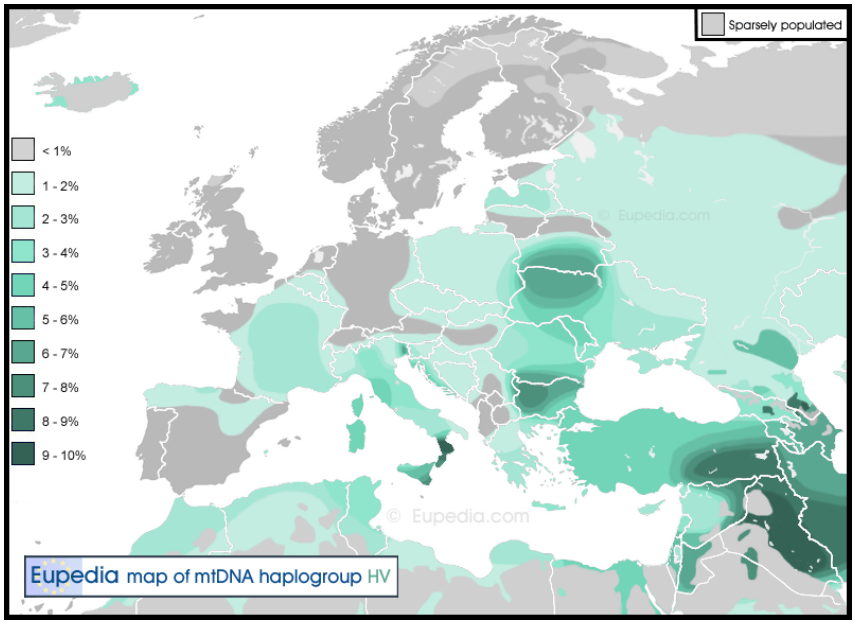

Figure 6. Identified HV haplogroup distribution in Europe and their relative frequency (eupedia.com)

and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

The bootstrap consensus tree inferred from 1000 replicates[20] is taken to represent the evolutionary history of the taxa analyzed (Figure 5). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach (Figure 4), and then selecting the topology with superior log likelihood value. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

The HV haplogroup represents the most fortuitous maternal lineage in Europe and the Near East (Figure 6). Recently, aDNA analyses suggested that some lineages within haplogroup HV, which was previously included as a whole in the "mitochondrial Neolithic package" as a specific marker of the Linear Pottery culture in central Europe, might have revealed some possible Palaeolithic signatures from European Late Glacial Maximum[21].

Conclusions

Genetic data for sample ST49 (M6 funerary context from the Stoicani "Cetățuia" necropolis) have revealed similarities with previously analyzed LBA samples from Romania which were assigned to Noua culture based on the typological characteristics of the grave goods[22].

Also, the HV haplogroup on the present-day Romania territory was, previously, identified in funerary contexts belonging to Early Neolithic (Starčevo-Criș culture), Middle Neolithic (Zau culture) and in two other LBA samples selected from Transylvania[23].

We caution that the cultural attribution of the funerary remains identified in different sectors of the Stoicani "Cetățuia" necropolis was done using only the typological characteristics of the grave goods, and that M6 funerary context does not contain any grave goods. Until now, no radiocarbon analysis was performed on the funerary remains, therefore the cultural assignation of the selected sample must be interpreted with care.

[20] FELSENSTEIN 1985.

[21] De FANTI *et alii* 2015.

[22] BOLOHAN *et alii* 2016.

[23] HERVELLA *et alii* 2015.

Acknowledgements. The financial support for this study was provided by *the PCCA 1153/2011 Nr. 227/01.10.2012 Genetic Evolution: New Evidences for the Study of Interconnected Structures. A Biomolecular Journey around the Carpathians from Ancient to Medieval Times (GENESIS)*. We would like to express our gratitude to the Dr Costel Ilie from "Paul Păltănea" Museum of History from Galați who provided the osteological sample.

References

BOLOHAN, N., M. CIORPAC, F. MĂȚĂU, L. GORGAN 2016. DNA analysis of a Noua Culture funerary context from Eastern Romania. In: *ArheoVest. Interdisciplinaritate în arheologie și istorie*, 631–639. Szeged.

BOROFFKA, N. 2013, Romania, Moldova, and Bulgaria, In: H. Fokkens, A. Harding (eds.), *The Oxford Handbook of the European Bronze Age*. DOI: 10.1093/oxfordhb/9780199572861.013.0047.

BURTĂNESCU, F. 2002. *Epoca timpurie a bronzului între Carpați și Prut cu unele contribuții la problemele perioadei premergătoare epocii bronzului în Moldova*. București.

De FANTI, S., C. BARBIERI, S. SARNO, F. SEVINI, D. VIANELLO, E. TAMM, E. METSPALU, M. Van OVEN, A. HUBNER, M. SAZZINI, C.M. FRANCESCHI, D.PETTENER, D. LUISELLI 2015. Fine dissection of human mitochondrial DNA haplogroup HV lineages reveals Paleolithic signatures from European glacial refugia. *PloS ONE* 10 (12), e014439. DOI: 10.1371/journal.pone.0144391.

FELSENSTEIN J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783–791. DOI: 10.1016/j.bse.2010.10.005.

GORGAN, L., M. CIORPAC, F. MATAU, N. BOLOHAN 2016. DNA Analysis of Late Bronze Age funerary context from Eastern Romania. *Annals of the "Alexandru Ioan Cuza" University, Section IIa, Genetics and Molecular Biology* 17 (4), 153–162. Online: www.gbm.bio.uaic.ro/index.php/gbm/article/view/1187.

HASEGAWA M., H. KISHINO, T. YANO 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22, 160–174. DOI: 10.1007/BF02101694.

HERVELLA, M., M. ROTEA, N. IZAGIRRE, M. CONSTANTINESCU, S. ALONSO, M. IOANA, C. LAZĂR, F. RIDICHE, A.D. SOFICARU, M.G. NETEA, C. De-La-RUA, 2015. Ancient DNA from South-East Europe reveals different events during Early and Middle Neolithic influencing the European genetic heritage. *PloS ONE* 10(6), p. e012881. DOI: 10.1371/journal.pone.0128810.

KUMAR S., G. STECHER, K. TAMURA 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33, 1870–1874. DOI: 10.1093/molbev/msw054.

MOTZOI-CHICIDEANU, I. 2011. *Obiceiuri funerare în epoca bronzului la Dunărea Mijlocie și Inferioară*, vol. I. București.

NEI, M., S. KUMAR 2000. *Molecular Evolution and Phylogenetics*. New York.

PETRESCU-DÎMBOVIȚA, M. 1953. Cetățuia de la Stoicani. *Materiale* 1, 13–155.