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գիտական աստիճանի հայցման ատենախոսության

ՍԵՂՄԱԳԻՐ

ԵՐԵՎԱՆ – 2021

NATIONAL ACADEMY OF SCIENCES OF THE REPUBLIC OF ARMENIA
INSTITUTE OF MOLECULAR BIOLOGY

MARIYA ALIK ANTONOSYAN

MOLECULAR INSIGHTS INTO THE REFUGIUM HYPOTHESIS FOR THE LESSER CAUCASUS DURING THE LAST GLACIATION

SYNOPSIS

of Dissertation Submitted for the Degree of
Candidate of Biological Sciences (PhD) in the Field of
03.00.03 – "Molecular and Cellular Biology"

YEREVAN - 2021

Ատենախոսության թեման հաստատվել է ՀՀ ԳԱԱ Մոլեկուլային կենսաբանության ինստիտուտի գիտական խորհրդում:

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Պաշտոնական ընդդիմախոսներ՝	կ.գ.դ., պրոֆ. Գալինա Գեորգիի Հովհաննիսյան
	կ.գ.դ. Մարինե Սեմյոնի Առաքելյան
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Ատենախոսության պաշտպանությունը տեղի կունենա 2021թ. հուլիսի 26-ին, ժամը 14^{00}-ին ՀՀ ԳԱԱ Մոլեկուլային կենսաբանության ինստիտուտում, 042 մասնագիտական խորհրդի նիստում (ՀՀ, 0014, ք.Երևան, Հասրաթյան 7):
Ատենախոսությանը կարելի է ծանոթանալ ՀՀ ԳԱԱ Մոլեկուլային կենսաբանության ինստիտուտի գրադարանում և http://www.molbiol.sci.am կայքում:

Սեղմագիրն առաքվել է 2021թ. հունիսի 14-ին:

042 մասնագիտական խորհրդի գիտական քարտուղար,
կենս. գիտ. թեկնածու՝ Գ.Մ. Մկրտչյան

Dissertation topic approved at the Scientific Council of the Institute of Molecular Biology NAS RA.

Scientific supervisor:	D.Sc., prof. Levon Michael Yepiskoposyan
Official opponents:	D.Sc., prof. Galina Georgi Hovhannisyan
	D.Sc. Marine Semyon Arakelyan
Leading organization:	Scientific Center of Zoology and Hydroecology, NAS RA

The defense of the dissertation will be held on 26 July 2021, at 14:00 at the session of the specialized council 042 acting in the Institute of Molecular Biology NAS RA (Hasratyan 7, 0014, Yerevan, RA).
The dissertation is available at the library of the Institute of Molecular Biology NAS RA and at the website http://www.molbiol.sci.am.

Synopsis was sent out on 14 June 2021

Scientific secretary of the specialized council 042
PhD G.M. Mkrtchyan

INTRODUCTION

Problem statement. The onset of the Last Glacial Maximum (LGM; 26,500 to 20-19,000 calibrated years before present (cal. BP; Clark et al., 2009) led to ecological restructuring, species redistribution and extinctions that shaped the current environment. During the LGM, thermophile plants and animals were confined to isolated temperate climate refugia (Hewitt, 2000; Provan & Bennett, 2008). In Europe, the Iberian, Apennine and Balkan peninsulas are the traditionally accepted southern refugia for temperate species and it is believed that after the LGM, the rest of the continent was recolonized from these areas by species moving northward as the climate warmed (Hewitt, 2000). Some authors suggest that along with major Mediterranean refugia other southern regions have played a key role in sheltering temperate biota during Pleistocene glaciations (Schmitt & Varga, 2012). Being climatically buffered by the Caucasus Mountains and benefiting from the ameliorating effects of the Black and Caspian Seas, the Lesser Caucasus might have served as such a biogeographical refugium throughout the Pleistocene (Fernández-Jalvo et al., 2016; Tarasov et al., 2000; Orth et al., 2002). Vertebrate fossils are an important source of information in understanding the prehistoric environment and ecological peculiarities. However, the accuracy of fossil identifications mainly relies on the easily observable morphological characteristics, making the classification of fragmented or taxonomically-mixed bone records challenging, if not impossible. However, various molecular strategies recently have been developed for those options when morphology-based identification proved problematic.

Over the past two decades, ancient DNA (aDNA) research has proved to be a useful complement to the morphological study of fossils; this approach is rapidly growing in popularity, accessibility, and applicability. Since its advent in the mid-1980s (Higuchi et al., 1984; Pääbo, 1985) the field of aDNA has brought powerful tools for studying the past. In particular, aDNA provides access to genomic data covering hundreds of thousands of years, allowing addressing evolutionary, ecological, social, and environmental questions, especially regarding the ways humans have interacted with other species and modified past ecosystems (Brunson & Reich, 2019). Ancient DNA research has made massive progress in its rather short history, extending greatly with the advent of next-generation sequencing technologies. Novel molecular approaches have made it possible to regularly acquire data from dozens of variable positions in the genome of increasingly diverse sources drawn from archaeological, palaeontological, and archival materials (Haouchar et al., 2014). Further, developments in aDNA sequencing have now enabled the mapping of entire nuclear genomes of fossils. The information acquired even from a single genome provide direct insights into the demographic history of past generations. Such data are often lacking in an area of study due to incomplete fossil assemblages and chronologies (Swift et al., 2019).

A necessary premise for aDNA research is the sufficient preservation of biomolecules. Cave systems represent an ideal environment for palaeontological investigation since they often contain relatively complete stratigraphic deposits coupled with stable environmental proxies, as minimal temperature and humidity fluctuations proved to be favourable for DNA preservation (Haouchar et al., 2014). Karin Tak cave is one of such unique palaeontological sites in the Lesser Caucasus, with environmental conditions that are optimal for biomolecules preservation,

potentially allowing molecular reconstruction of the prehistoric ecosystem of this region to be investigated for the first time. The cave contains Late Pleistocene to Holocene sediment infill together with hominid remains, obsidian stone tools and contemporaneous flora and fauna (Antonosyan et al., 2020; 2021). As is commonly the case, excavations at Karin Tak have mainly provided highly fragmented and morphologically undiagnostic bones, which are not informative for reconstructing prehistoric faunal dynamics. The excavations yielded only a handful of bones that were taxonomically identifiable based on morphological traits. In this context, aDNA analysis of the fossils represents an effective complementary tool for detailed mapping of faunal diversity. One of these new aDNA methods, bulk bone metabarcoding, was applied here to reconstruct the taxonomy of fossil assemblage recovered from Karin Tak cave.

Aim and objectives: The principal aim of the project is to estimate ancient faunal diversity and its dynamics in the Lesser Caucasus and test the refugium hypothesis during the Last Glaciation using a combination of traditional morphological methods and novel molecular genetic tecniques of taxonomic identification.

The specific objectives are as follows:

- Assess the preservation of ancient proteins and DNA in the fossil bones acquired from Karin Tak cave based on molecular screening of collagen and DNA.
- Develop an accurate chronological sequence of sediments via assessing the ages of ancient specimens recovered from different stratigraphic layers.
- Reconstruct faunal composition and its structural dynamics through time based on traditional morphological methods and molecular genetic metabarcoding techniques.
- Test the refugium hypothesis for the Lesser Caucasus relaying on dynamics of faunal diversity from pre-glacial to glacial epochs.

The scientific and practical significance of the results: Recent advances in molecular technologies have revolutionized archaeological sciences and contributed to the development of a novel field of molecular archaeology. Currently, via detailed exploration of fossil samples, using innovative methods of molecular archaeology, we can address questions on human prehistoric migrations (Skourtanioti et al., 2020), adaptation mechanisms (Rivollat et al., 2020), subsistence patterns (van de Loosdrecht et al., 2020), climate changes impacting biodiversity (Seersholm et al., 2020), as well as many others that remained unexamined while using traditional approaches.

Until recently, no prehistoric sites preserving molecular data were discovered in the Lesser Caucasus. This might be the reason why Armenia remained isolated from this innovative scientific field. With the latest discovery of Karin Tak cave that preserves ancient biomolecules sufficient for genomic screening, we are entering the stage of applying state-of-the-art molecular tools to fossil bones.

Karin Tak is the only site in the region where favourable conditions for the preservation of aDNA and ancient proteins allow studying the genetic makeup of our ancestors and identification of ecosystems (Margaryan et al., 2017; Antonosyan et al., 2019, 2021). Before, all the knowledge on the Late Pleistocene fauna of the region was based on morphological identification of fossils, whereas non-identifiable fragments were considered as waste and discarded. This approach brings about the loss of a huge amount of valuable information, which,

in its turn, creates a bias in the patterns of ecological reconstruction. The study of Karin Tak addresses this issue by genetic identification of morphologically non-diagnostic bone fragments thus complementing our understanding of ancient biodiversity in the Lesser Caucasus. In addition, this approach allows the reconstruction of regionally extinct species and those previously not identified in the regional Late Pleistocene sites.

The site contains a continuous sequence of cultural layers dated from the Late Pleistocene to Holocene, rich with numerous human and animal bones, seeds, stone tools, and pottery. Karin Tak sediments dated between ca. >42-24,000 cal. BP are associated with the last phase of interstadial Marine Isotope Stage 3 (MIS 3; 57-29,000 BP) with a relatively warm and humid climate, and coincide with the beginning of the last glacial cycle MIS 2 (29-14,000 BP), thus marking the onset of the LGM (26-19,000). These results point out that the Karin Tak fossil assemblages belong to chronological layers embracing the onset of the LGM that allows testing the impact of LGM and answering the question of whether the Lesser Caucasus served as a refugial zone during the last glaciation for a bulk of species.

Our study addresses the Late Pleistocene vertebrate faunal diversity in Lesser Caucasus based on morphological and genetic identification of fossil bones from Karin Tak cave. For the first time in this under-studied region, a bulk bone metabarcoding genetic approach was applied to complement traditional morphology-based taxonomic identifications that are hampered by highly fragmented fossil bones. Excellent aDNA preservation allowed for a successful species identification of many bone remains and improved palaeoenvironmental interpretations for the region. Genetic screening of vertebrate fossils has revealed a high diversity of animal taxa inhabiting the region between ca. >42,000 and 25,683-24,803 cal. BP. The reconstructed taxonomic assemblage comprises 29 taxa, including 11 mammalian and three avian families currently inhabiting the area, together with a few taxa regionally extinct today. Based on 14C chronology, the taxonomic assemblage indicates faunal continuity in the region during the Late Pleistocene. This suggests that the transition between warm and humid MIS 3 and cold and arid MIS 2 with the onset of the LGM did not cause a dramatic change in the faunal makeup of the region, and during the considered timespan, Karin Tak cave was located at the boundary between arid subtropical and humid climate regions, a pattern preserved till the modern days (Antonosyan et al., 2019). Further exploration of the cave will include a larger number of animal fossils and botanical remains to thoroughly test the hypothesis of refugium for the Lesser Caucasus.

Approbation. Proceedings of the dissertation have been presented at “1st virtual conference for woman archaeologists and palaeontologists”, online; “EMBO/EMBL Symposium: Reconstructing the Human Past – Using Ancient and Modern Genomics”, Heidelberg, Germany; "International conference Caves as Natural and Cultural Monuments", Yerevan, Armenia; "8th Postgraduate Zooarchaeology Forum (PZAF)", Yerevan, Armenia; "7th Postgraduate Zooarchaeology Forum (PZAF)", Palermo, Italy; "2-nd International Young Scientists Conference on Biodiversity and Wildlife Conservation Ecological Issues", Tsaghkadzor, Armenia; “Women Achievements in Biological Sciences, II International Conference” Yerevan, Armenia and "Biological Diversity and Conservation Problems of the Fauna-3", Yerevan, Armenia.

Publications. The main results of the dissertation are published in 4 papers and 9 abstracts of presentations at local and international scientific conferences.

Structure. The dissertation comprises 114 pages of computer-formatted English text, including 12 tables and 28 figures, and consists of the following sections: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusion, Inferences, and References (including 208 sources).

MATERIALS AND METHODS

Exploration site. Karin Tak cave is an undisturbed site located at the southeastern end of the Lesser Caucasus mountain range, in the midst of the lush Karintak forest, on the right bank of the Karkar River. The site derives its name from a nearby village, situated in a valley ca. 600 m beneath the cave. Karin Tak is a limestone cavern that comprises two separate passages conventionally termed Cave 1 and Cave 2. The currently explored extent of Cave 1 includes six chambers. Stalactites, stalagmites, and flowstone are observed in the rear of the cave. The site is still active and wet today, it exhibits minimal seasonal temperature fluctuations: 13^{o}C in summer and 8^{o}C during winter. Inner chambers are devoid of any daylight penetration. These conditions are conducive to ancient biomolecules preservation. The geology of the site displays that Karin Tak was an attractive natural shelter for animals and humans, with a closely located water and rich food resources due to the dense forest surrounding the cave. Additionally, the stratigraphy reveals that the site acted as a natural repository for the accumulation of sediment containing a four-meter infill of archaeological layers that have great potential to reveal the environment of the region through large chronological succession.

Fossil material. The fossil material presented here was recovered from the inner chamber of Cave 1 during the 2015 exploratory field season. In 2015, a two-meter long and 0.5 m wide test pit approximately ca. 46 meters from the cave mouth was excavated, divided to one-square meter areas (A and B) to a depth of 60 cm. To minimize bias in the chronology, which might be caused by mixed sediments, the analyzed bones were sampled exclusively from the area A, where all radiocarbon-dated specimens were collected. The bones acquired during the field season were excavated *in situ* and recorded by stratigraphic position following standard palaeontological methods. Contamination reduction methodologies (Llamas et al., 2017) were employed during all phases of fieldwork to minimize pollution of fossils with exogenous DNA.

Collagen extraction and mass spectrometry. Bone powder was demineralized in HCl and gelatinised by incubation in AmBic for 3 h. Gelatinized samples were centrifuged and trypsin solution was added to the supernatant and subsequently incubated. The peptides were purified using a C18 ZipTip. Sample solutions were diluted with TFA, and mixed with an equal volume of α-cyano-4-hydroxycinnamic acid solution. The mixture was spotted on a Bruker ground steel plate in triplicate. Samples were measured using an Autoflex Speed LRF Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI TOF) Mass Spectrometer (Bruker). The resulting mass spectra were analyzed using m Mass (V.5.5.0) together with Byonic Viewer (V3.4-55-g551bce936c). This screening was performed at the Department of Archaeology, Max Planck Institute for the Science of Human History (Jena, Germany).

Morphological identification. All bones were examined and separated into two categories: identifiable and unidentifiable fragments. Maximum length and width were measured. Anatomical identification and taxonomic affiliation of remains were carried out based on osteological catalogues. The number of identified specimens (NISP), the minimum number of individuals (MNI) and minimum number of skeletal elements (MNE) were determined. All specimens were systematically examined for bone-surface modifications using a digital stereoscopic light microscope with 3.5x to 90x magnification (AmScope SM 2TZ).

Sample preparation and DNA extraction. Five bulk bone collections (total of 250 bones) from the Karin Tak cave were analyzed at the TRACE (Trace Research Advanced Clean Environment) aDNA facility at Curtin University, Western Australia. Morphologically indistinct fossils were subsampled into pools of 50 bone fragments and ground to bone powder. DNA was extracted from pools by dissolving bone powder in a lysis buffer followed by incubation overnight. Next, samples were centrifuged and concentrated DNA was purified using a MinElute polymerase chain reaction (PCR) Purification Kit (Qiagen).

Amplification and sequencing. DNA was amplified using two sets of primers, 12Sv5 and Mam16S targeting a short section of the mitochondrial 12S and 16S rRNA gene regions, respectively, following the methods of (Seersholm et al., 2018). DNA reads were sequenced on the MiSeq platform and obtained raw FASTQ files were filtered using Obitools.

Bioinformatical and statistical analyses. For taxonomic assignments, filtered unique reads (ASVs) were queried against the NCBI NT database using BLAST. BLAST files were parsed using the blast_getLCA algorithm, which assigns reads to the lowest common ancestor. Thereafter, raw taxonomic assignments were individually assessed by an experienced operator, correlating each assignment with data on species known to inhabit the area and data on relevant species missing from the reference database. The dendrogram was generated based on the NCBI taxonomy of the species identified with BBM used the script: create_tree_from_curated_list.py (https://github.com/frederikseersholm/blast_getLCA).
Correspondence analysis was carried out in R using the Vegan package (https://cran.r-project.org/web/packages/vegan/index.html).

Dating. The chronology is based on Accelerator Mass Spectrometry (AMS) 14C ages, performed in Direct AMS lab, Washington, USA, and CHRONO centre and Queens University Belfast, UK. Four stratigraphic positions in Pit 1 were dated by using four AMS 14C measurements on medium-sized mammal bones representing different depths (1-47 cm) of the excavated area A. The fossil remains included single skeletal elements and partial dentary fragments.

RESULTS AND DISCUSSION

Assessment of biomolecules preservation in fossil bones

An essential prerequisite for ancient molecular research is the sufficient conservation of biomolecules in fossils. Many taphonomic factors influence the preservation of organic material at the macroscopic (morphological peculiarities) or molecular (proteins, DNA) level of organization. Therefore, good biomolecular preservation is more of an exception than a rule. and depends on two linked drivers: environment and time (Brothwell & Pollard, 2005). Cave

systems often serve as adequate sources for ancient molecular research since they typically preserve relatively complete stratigraphic sequences together with stable environmental conditions favourable for the conservation of biomolecules. Karin Tak is one such cave that contains Late Pleistocene fossiliferous sediments and is described by stable conditions that support chemical conservation (Antonosyan et al., 2019, 2021; Margaryan et al., 2017).

At first, molecular preservation at Karin Tak was checked through the assessment of collagen, the main protein of bone tissues. In particular, 100 non-diagnostic bones representing different stratigraphic layers were screened for collagen preservation using MALDI TOF mass spectrometry. The analysis revealed high concentration of collagen in 80% of studied bones, relative to the spectra of modern samples of bovids from Africa (Figure 1). The result indicates that collagen preservation in Karin Tak is sufficient for further radiocarbon dating and proteomic classification. This allows suggesting that DNA preservation at the site might be equally high.

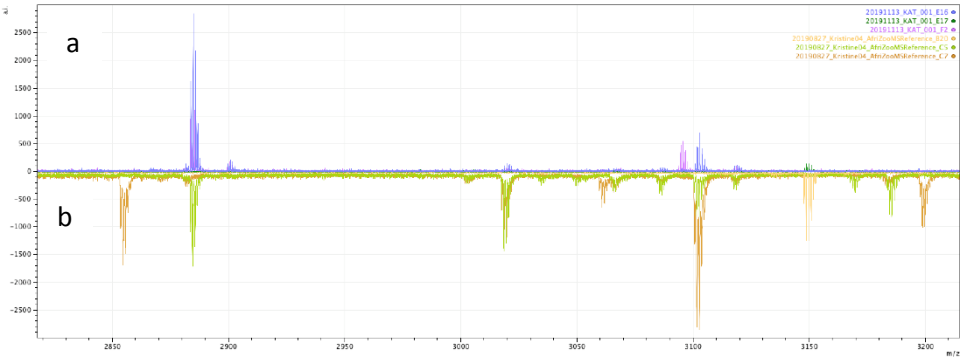


Figure 1. Peptide mass fingerprint spectra for collagen extracted from Karin Tak fossils (a) in comparison with modern reference (b). The y-axis – ion intensity, and the x-axis – the mass-to-charge ratio (m/z).

At the same time, 25 bones from different layers were screened for ancient mtDNA preservation. For the first stage, DNA was extracted and amplified with further assessment of libraries using gel electrophoresis. Figure 2 displays the distribution of DNA bands under UV light. 20 samples are described with bright light indicating high preservation of DNA in bones.

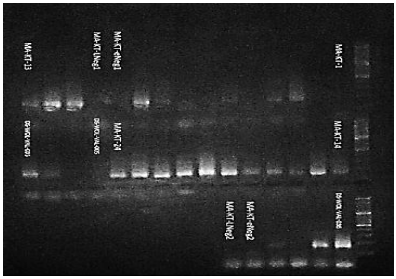


Figure 2. Gel electrophoresis profile of mtDNA of 25 bones from different layers of Karin Tak.

Thereafter, five samples were quantified on Agilent Bioanalyzer to estimate the concentration of DNA particles. Once again, the electropherogram indicates a high concentration of amplified DNA fragments that vary in length, with 187 bp length fragments of the highest concentration (Figure 3). Further sequencing revealed varying endogenous DNA content (0.1-56%) that is sufficient for subsequent genetic screening of specimens

Relying on these results we have all grounds to claim that Karin Tak is a unique regional site where exceptional preservation of ancient proteins and DNA ensures effective in-deep molecular analysis of acquired fossils.

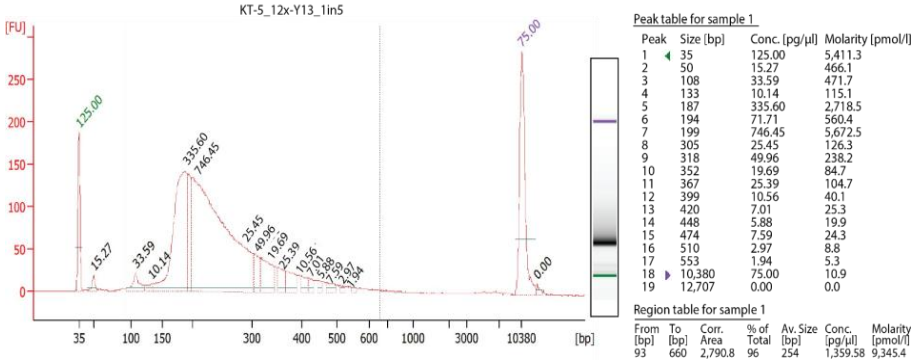


Figure 3. Electropherogram and gel-like image of the selected sample from Karin Tak. The y axis represents fluorescence units (FU), and the x-axis represents base-pair sizes.

Results of radiocarbon dating

Four stratigraphic positions in Pit 1 were dated by using four AMS 14C measurements on medium-sized mammal bone. Bones applied for AMS radiocarbon dating contained 3-5% collagen and yielded fair to moderate collagen pseudomorphs. The dating indicated an age from 25,683 to 24,803 to 34,486-33,657 cal. BP for the upper 40 cm of stratigraphy while the age of sediments 47 cm below the modern cave floor is beyond the limit of radiocarbon dating, >44,000 years. These results point out that the studied fossil assemblages belong to chronological layers embracing the MIS 3 interstadial and onset of the LGM. It allows testing the impact of LGM and address the question of whether the Lesser Caucasus served as a refugial zone during the Last Glaciation for a bulk of species.

Main characteristics of the fossil assemblage

A total of 1,749 complete and fragmentary bone elements were recovered from the 2015 excavation in Karin Tak, and 835 of these were assigned to area A.

Though the recovered bones are remarkably well preserved physically, the main feature of the Karin Tak fossil assemblage is a high percentage of bone fragmentation. The principal factors causing fragmentation may include a combination of the chimney collapse, post-burial sediment compaction, together with biological agents such as humans and/or carnivores. The excavated fragments differ widely in size and distribution (Figure 4).

The morphological identification of the animal remains was greatly hampered by their high fragmentation. Of this assemblage, only about 50 bones (8.7% of all fragments) bear diagnostic morphological characteristics and therefore were assigned anatomically and/or taxonomically, the rest of the fossil collection was screened using a novel genetic approach of identification.
Identification of all morphologically diagnostic bones revealed six taxa belonging to five mammalian families (Figure 5) representatives of which are currently inhabiting the region. The NISP and MNI were used to quantify the relative abundance of identified taxa through time. In all layers, ungulates are the most common groups with sheep/goat dominating the record**.**

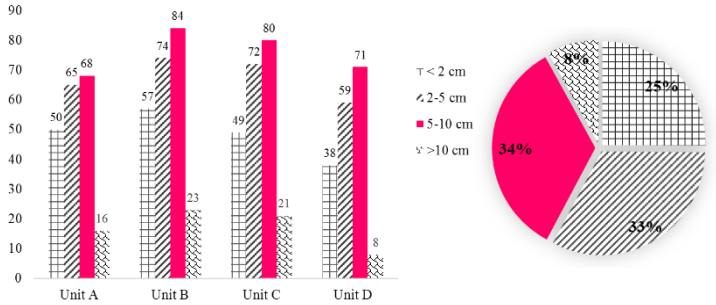


Figure 4. Proportions of different size groups of fossil bones accoutered in different units of Karin Tak cave referred to the total number of excavated fossils (835).

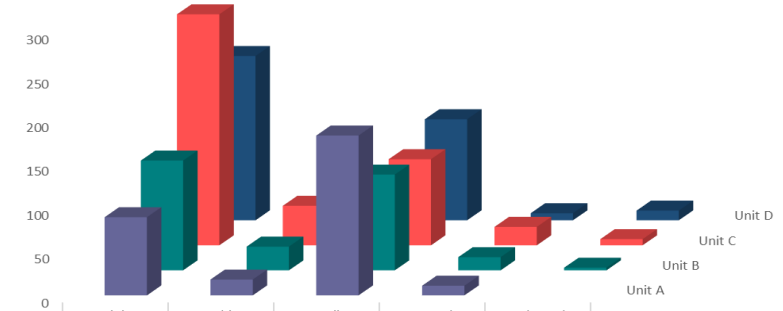
			MIS 3		early MIS 2		
			> 42 ka	34-33 ka	27-26 ka	25-24 ka	
Bovidae	Caprinae	*Capra/Ovis*	✓	✓	✓	✓	✓
	Bovinae	*Bos*	✓		✓	✓	✓
Cervidae	Caprinae	*Capreolus capreolus*			✓		
Equidae	*Equinae*	*Equus*	✓				
Canidae	Caninae	*Canis lupus*		✓		✓	
Mustelidae	Melinae	*Meles meles*			✓		
			40-50cm	30-40cm	0-10cm	10-20cm	20-30cm

Figure 5. Morphologically identified taxonomic composition.

All bones, both diagnostic and non-diagnostic fractures, were systematically screened for surface modifications. Figure 6 summarizes these taphonomical features of fossils registered at different units. Overall, the morphologically reconstructed set of taxa is not sufficient for a comprehensive description of ancient biota. To address this gap and obtain a more complete pattern of ancient faunal diversity we endeavoured to acquire additional information from the fragmented fossils using molecular genetic techniques.

Results of the genetic identification of fossil bones

To complement the morphology-based data and expand our knowledge on the Late Pleistocene faunal composition and dynamics, we genetically identified the taxa from five bulk bone pools comprising 250 morphologically non-diagnostic bone fragments. We were able to recover aDNA from all powder samples using two mitochondrial metabarcoding assays targeting vertebrate 12S and mammalian 16S rDNA.



	staining	craching	trampling	cut marks	tooth marks
Unit A	89	18	182	11	
Unit B	125	27	109	15	3
Unit C	263	45	98	21	7
Unit D	187	37	115	8	11

Figure 6. The abundance of bone surface modifications in the stratigraphic layers of Karin Tak.

Endogenous DNA was amplified generating products of 100–104 bp and 90–96 bp respectively. The Next-generation sequencing of DNA yielded a total of 157,787 reads after filtering (34,143 reads per sample on average), corresponding to 88 amplicon sequence variants (ASVs) from the 12S assay and 87 ASVs from the 16S assay (175 ASVs in total). Of these, 107 amplicon sequence variants were assigned to a taxonomic node at the family level or below (Figure 7). The majority of these sequences were assigned with high confidence to a species level (13 specimens), however, in some cases, identification was restricted to a genus (nine taxa), subfamily (three taxa) or family (1 taxon) scale.

The genetic screening of fossil bones revealed a highly diverse composition of vertebrate species across all layers dated between ca. >42,000 and 24,000 cal. BP. We identified representatives of twenty-one genera, belonging to eleven mammalian and three avian families. The dendrogram in Figure 8 demonstrates the overall biodiversity of screened bulk bone samples. In contrast to morphological identification, the genetic screening revealed a great variety of taxa that are currently inhabiting the region, together with regionally extinct ones. In addition, the genetic approach allowed to identify several large-bodied mammals that have not been previously described from the Late Pleistocene sites of the region (Figure 9 A).

Large mammals predominate the record, with eight families representing 18 taxa (~66%). Ungulates are the most commonly registered group dominated by a wild goat and sheep registered in all layers. Other ungulates such as roe and red deer are less common. Carnivores are also markedly encountered in the record with the predominance of bears (*Ursus arctos* and *U. tibethanus*). Abundance of bear tooth marks on bones suggests that these predators played a key role in the formation of the fossil assemblage. Micromammals (rodents and bats) considerably less common in our dataset are represented by isolated specimens of six taxa (~22%). This pattern occurs due to sampling bias since complementary micro mammalian bones were not selected for the genetic screening.

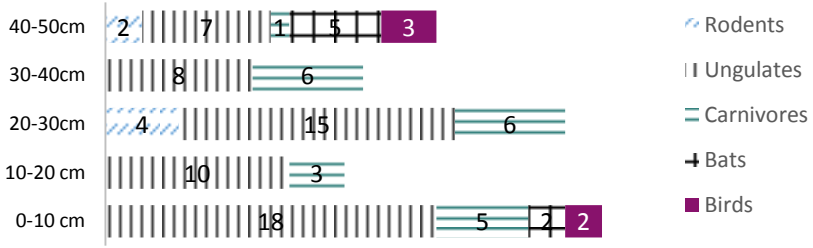


Figure 7. Number of ASVs assigned to genus level per layer.

The reconstructed avian fauna comprises three extant taxa representing, in aggregate, ~11% of the identified assemblage. This could be explained by sampling bias as well as by differential preservation of fossils (fragile and hallow bird bones are less common in archaeological material). Additionally, the genetically recovered composition includes four regionally extinct taxa: spotted hyena, Asiatic black bear, goitered gazelle, and Ciscaucasian hamster.

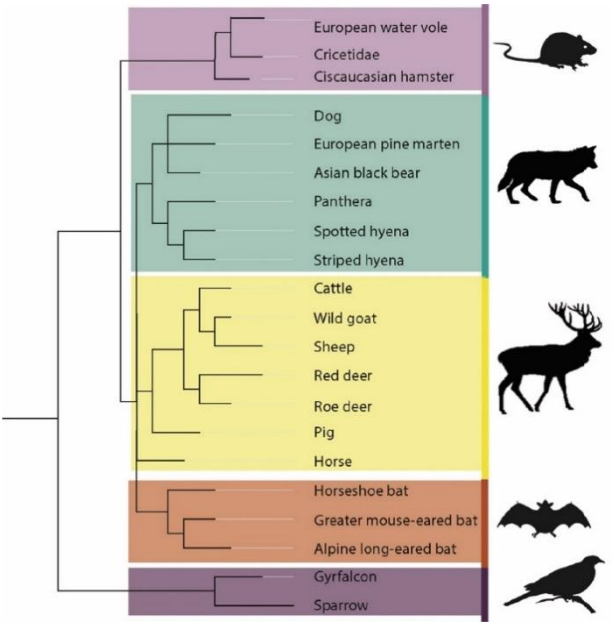


Figure 8. Dendrogram of genetic diversity identified through DNA metabarcoding of all samples.

In a whole, the genetically identified fauna does not appear to indicate major contrasts between the different layers and it agrees with and complements the morphology-based reconstruction. Recovered MIS 3 assemblage is rich with arid-adapted ungulates and their predators together with forest inhabitants. In the same manner, the early MIS 2 collection comprises a similar set of both arid and humid environment occupants.

To visualize genetic and morphological variation in the abundance of the identified taxa across different periods, correspondence analysis was applied (Figure 9 B). The results revealed four clusters of taxa associated with different periods; in all clusters, the proportions of forest adapted and arid associated taxa are almost equal. The only exception is the >42,000 cluster, where the taxa are associated with a wide range of environments together with a single species (*Arvicola amphibious*) indicating a humid climate. Thus, the analysis did not reveal a clear clustering of forest vs arid environment associated genera through the examined time.

Summing up, the taxonomic assemblage of Karin Tak indicates general continuity in faunal composition of the region during the Late Pleistocene. Moreover, the proportions of taxa associated with dry and forested environments are almost equal in all chronological layers. This allows suggesting that between ca. 42,000 and 24,000 years ago the cave was close to the boundary between arid subtropical and humid climatic regions (with the latter supporting forests), a pattern similar to the present environment of the site.

Karin Tak finds in a regional context

The assemblage of Karin Tak is supplemented by the number of neighbouring contemporaneous sites of Ortvale Klde, Satsurblia and Dzudzuana in Georgia, and Hovk-1, Kalavan-2 and Aghitu-3 in Armenia. Comparison with the faunal assemblages of these regional Late Pleistocene sites revealed many mammalian species similar to those from Karin Tak. The cave deposits at Aghitu-3 have yielded both arid zone (*Ovis, Capra, Equus, Bos/Bison*) and forest (*Cervus elaphus, Vulpes vulpes, Sus scrofa*) associated mammals from the 39-24,000 cal. BP horizons. Similarly, Hovk-1 Unit 4 (35,000 cal. BP) assemblage is dominated by forest taxa (*V. vulpes, C. elaphus, Capreolus capreolus, Meles meles,* and *Martes foina*) with few arid area representatives (*Capra aegagrus* and *Lepus europaeus*). Likewise, the faunal assemblages of Georgian sites Dzadzuana (Unit D, 35-32,000 cal. BP and Unit C, 27-24,000 cal. BP), Satsurbia (Layers B/III and B/II at 26 -24,000 cal. BP) and Ortvale Klde (Layers 4 at 40-26,000 cal. BP and 3 at 26-22,000 cal. BP) are mainly dominated by open-landscape taxa (*Capra, Ovis, Equus,* and *Bos/Bison*) together with forest associated ones such as *U. arctos, V. vulpes, C. elaphus,* and *C. capreolus*.

The recovered large mammalian diversity indicates general continuity in the composition of fauna in the region through the whole terminal Pleistocene with most of the species persisting through the whole stretch of the Last Glaciation. However, taking into account that large mammals occur in a relatively wide range of environments, it is worth focusing on micromammals that have narrow ecological tolerance limits and can act as bioindicators for environmental conditions. When we look at micro mammalian diversity through time, once again we notice distinct continuity in faunal composition. Visualization of environmental preferences of micro mammals based on moisture gradient illustrates that during LGM xeric as well as mesic species were inhabiting the region.

In both MIS 3 and MIS 2 collections, the proportions of small mammals associated with steppes are noticeably higher. Most probably that these species were captured in open grasslands by predators in a setting where forests are located in the vicinity of steppes.

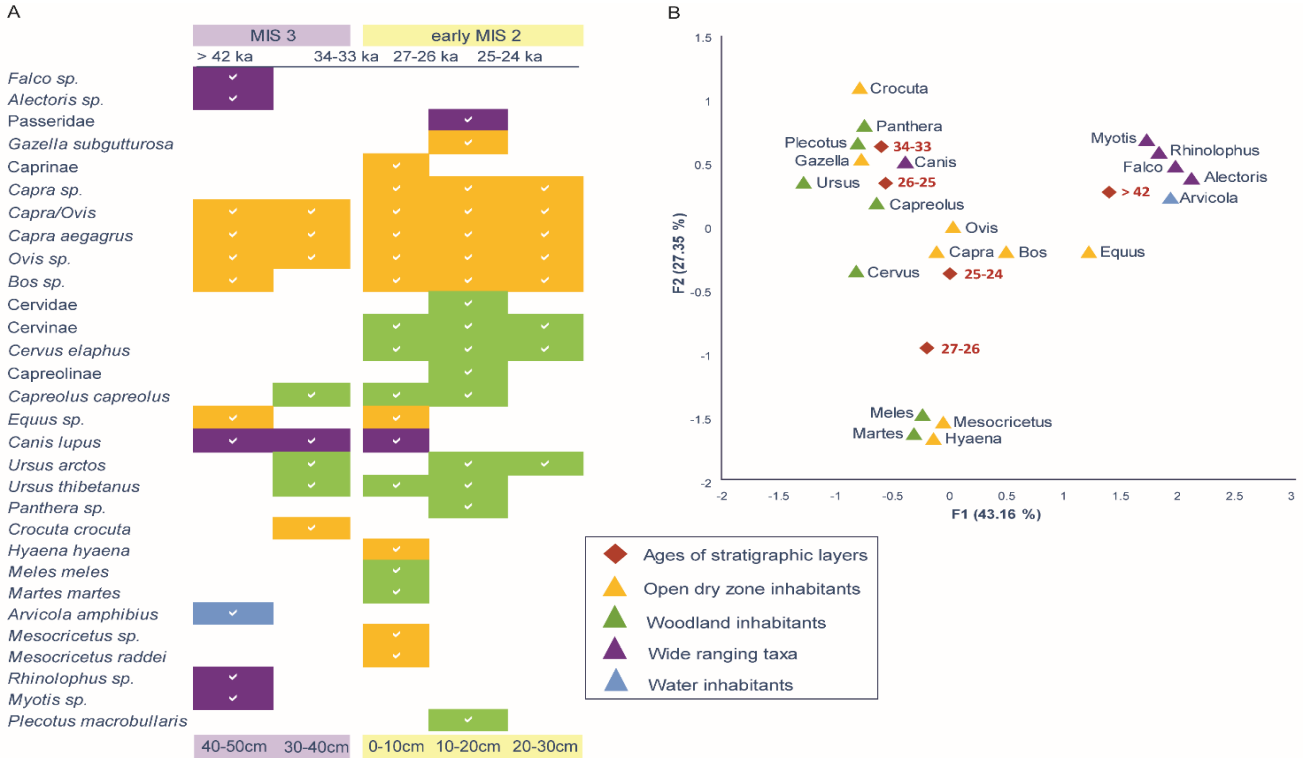


Figure 9. (A) Taxonomic composition of samples identified by morphological and bulk bone methods in different time periods; (B) visualization of the results of correspondence analysis based on the abundance variation and environmental preferences of the genera through time.

Similarly, the correspondence analysis based on the abundance variation of micromammals through time did not discern a clear clustering of forest and arid environment associated taxa in the examined time period (Figure 10).

These results support continuity of the same biota from MIS 3 to early MIS 2, with species distinctive to the broadleaved forests and arid steppe environments. Additionally, most of the regionally extinct species persisted the onset of the LGM being last registered in late LGM archaeological horizons. These late-survived taxa provide additional support to the suggestion that the onset of LGM did not cause a dramatic turnover in the faunal composition of the region thus indicating that the Lesser Caucasus was a climatically and ecologically stable region despite significant global climatic changes during the Last Glaciation.

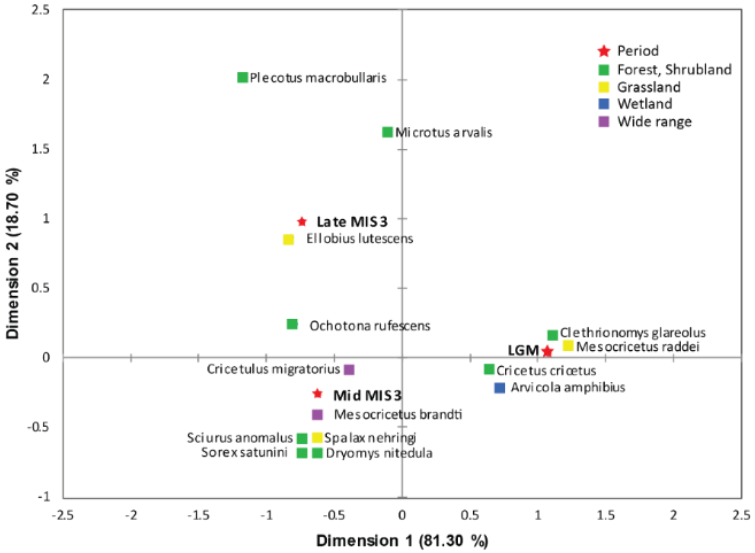


Figure 10. Vizsualization of the results of correspondence analysis based on the abundance variation and environmental preferences of the micro mammalian species through time.

Additionally, the patches of mesophilic Cenozoic plants are currently sporadically dispersed between the Black and Caspian Seas (Мулкиджанян, 1967), indicating the presence of spatially confined multiple refugia, where animals survived the cold and aridity of the glacial period.

Furthermore, the faunal and floral elements of Azokh cave (ca. 36,000 m distance from Karin Tak) provide detailed information on the Middle Pleistocene environment. The large mammals (van der Made et al., 2016) and charcoal (Allué, 2016) indicate deciduous woodland conditions, while small mammals (Parfitt, 2016), amphibians and reptiles (Blain, 2016) point to an open steppe environment for the region, thus demonstrating woodland area in the vicinity of the cave, and open steppe areas not far away. On the whole, the results of excavations at several regional sites provide strong evidence that the Lesser Caucasus served as a refugium during the Middle

and Late Pleistocene when the area was surrounded by arid, hyper-arid and periglacial landscapes, with extensive alpine glaciation in surrounding mountains.

The biodiversity richness, high level of endemic and hybrid taxa further support the presence of relatively stable and climatically favourable conditions, as well as the absence of permafrost in the area through the Pleistocene. These pieces of evidence provide a solid support to the increasing recognition of the southeastern Lesser Caucasus as a climatically and ecologically stable region at least since the Middle Pleistocene despite significant global climatic changes.

CONCLUSION

Located in the crossroad between Africa, Europe, and Asia, the Lesser Caucasus region served as a natural passage through which early hominins and fauna have followed during their migration from Africa to Eurasia since the Palaeolithic. So far, it remains hotly debated whether the region acted as a natural shelter (refugium zone) for thermophile biota during the Last Glaciation. Though the human and environmental history of this area is now being extensively explored, the complete picture of past environment and impact of the LGM on the ecology of the area remains unresolved. The present study contributes to our understanding of ancient biodiversity and extinction processes in the region during the MIS 3-MIS 2 transition based on morphological and molecular identification of fossil bones recovered from Karin Tak cave.

The main feature of the Karin Tak fossils assemblage is a high percentage of bone fragmentation. From the assemblage only ca. 50 bones bear taxonomically significant peculiarities enabling their classification to only six taxa belonging to five mammalian families. The morphologically reconstructed set of taxa is insufficient for a comprehensive description of ancient faunal composition in the area. Hence, to obtain an overwhelming pattern of the ancient faunal diversity of the region we acquired additional information from fragmented fossils using a modern molecular technique. Specifically, we genetically identified taxa from five bulk bone pools comprising 250 morphologically non-diagnostic bone fragments. The genetic screening revealed a high faunal diversity between ca. >42,000 and 24,000 cal BP. The genetically recovered taxonomic composition is rich and diverse and mainly consists of extant wild species together with regionally extinct ones. A total of 27 different taxa, represented by 11 mammalian and three avian families were identified.

The reconstructed MIS 3 assemblage is rich with arid-adapted ungulates and their predators, wolves and hyenas, together with forest inhabitants such as deer and bears. The early MIS 2 collection comprises a similar set of both arid and humid environment occupants. The MIS 2 assemblage also includes species typical for forested zones and open dry landscapes as gazelle, hyena, and hamster.

On the whole, the taxonomic assemblage reflects general continuity of warm adapted faunal composition in the region during the Late Pleistocene, with only a few extinct taxa. All units contain groups commonly associated with interglacial environments. This allows suggesting that cold and arid MIS 2 conditions did not cause a dramatic change in faunal makeup, and between ca. 42,000 and 24,000 years ago the cave was close to the boundary between arid subtropical and humid climate regions, a pattern similar to the present days.

This result, along with the finding of late-surviving taxa, demonstrates that during the MIS 3-MIS 2 transition, the region sheltered a wide range of animals of the temperate biota.Altogether, the outcomes argue in favour of the assumption that during the Glacial Maximum, the Lesser Caucasus lowlands persisted conducive climatic pockets backed by the Greater Caucasus mountain range that contributed to the preservation of warm temperate climate in the region, which served as a refugium to shelter warm adapted animal species.

INFERENCES

1. Faunal bones acquired in Karin Tak cave dated back to >42,000-24,000 years ago are characterized by an exceptional rate of preservation of proteins and DNA, which are the oldest ancient biomolecules so far recovered in the Lesser Caucasus region.
2. Molecular genetic screening of fossil specimens allowed establishing the taxonomic composition of about 90% of the studied collection, while only 6% of the assemblage was possible to determine morphologically. In most of the cases the molecular genetic approach ensured identification at a species level.
3. The molecular and phylogenetic study of the acquired Late Pleistocene fossils allowed restoring the rich and diverse taxonomic composition of the site which comprises 27 taxa (11 mammalian and three avian families).
4. The recovered faunal diversity is mainly represented by warm adapted taxa that are currently inhabiting the region with an exception of four regionally extinct species.
5. Faunal continuity and the prevalence of temperate biota in the Lesser Caucasus during the Late Pleistocene suggests that the transition between warm and humid MIS 3 and cold and arid MIS 2 did not cause a dramatic change in the biodiversity of the region thus supporting the refugium hypothesis for the area.

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ԱՆՏՈՆՈՍՅԱՆ ՄԱՐԻՅԱ ԱԼԻԿԻ

ՎԵՐՋԻՆ ՍԱՌՅԱՊԱՏՄԱՆ ԺԱՄԱՆԱԿ ՓՈՔՐ ԿՈՎԿԱՍԸ ՈՐՊԵՍ ՌԵՖՈՒԳԻԱԼ ԳՈՏԻ ՎԱՐԿԱԾԻ ՄՈԼԵԿՈՒԼԱՅԻՆ ՀԻՄՈՒՆՔՆԵՐԸ

ԱՄՓՈՓԱԳԻՐ

Բանալի բառեր. հնագույն սպիտակուցներ, հնագույն ԴՆԹ, ԴՆԹ մետաբարկոդավորում, ֆաունայի մոլեկուլագենետիկական վերակառուցում:

Տեղակայված լինելով Աֆրիկայի, Եվրոպայի և Ասիայի միջև աշխարհագրական միջանցքում՝ Հարավային Կովկասը ծառայել է որպես բնական անցուղի, որով, սկսած հին քարի դարից, Աֆրիկայից Եվրասիա են գաղթել հնագույն մարդիկ և կենդանիներ: Արդյո՞ք տարածաշրջանը վերջին սառցապատման ժամանակ ծառայել է որպես բնական ապաստարան (ռեֆուգիալ գոտի) ջերմասեր տեսակների համար: Թերևս այս հարցը առ այսօր մնում է վիճահարույց:

Մոլեկուլային տեխնոլոգիաների վերջին նվաճումները հեղափոխություն են կատարել գիտության մի շարք բնագավառներում՝ հիմք ծառայելով նոր ճյուղի՝ մոլեկուլային հնէաբանության զարգացման համար: Ներկայում մոլեկուլային հնէաբանության նորամուծական մեթոդների միջոցով բրածո մնացորդների մանրակրկիտ ուսումնասիրության շնորհիվ հնարավոր է բացահայտել հնագույն կենսամիջավայրի հետ կապված մի շարք հարցեր, որոնց պատասխանները հնարավոր չէ ստանալ ավանդական մոտեցումներով: Մինչև վերջերս Հայաստանը ներառված չէր գիտական այս առաջադեմ ոլորտում, քանի դեռ տարածաշրջանում չէր հայտնաբերվել հնագույն կենսամոլեկուլների բավարար պահպանվածության բրածո նյութ պարունակող նախապատմական հնավայր: 2011 թվականին Արցախի Քարինտակ գյուղի շրջակայքում այդպիսի քարանձավի հայտնաբերումն արմատապես փոխեց իրավիճակը՝ հայ կենսաբանների և հնագետների համար բացելով արդի տեխնոլոգիաների միջոցով մարդկանց և կենդանիների հնագույն մնացորդների մոլեկուլային ուսումնասիրման նոր շրջափուլ:

Հնավայրը պարունակում է միջին քարից մինչև պղնձի դարաշրջաններով թվագրվող մշակութային շերտերի շարունակական հաջորդականություն, հարուստ է մարդկային և կենդանական ծագման բազմաթիվ ոսկորներով, սերմերով, քարե գործիքներով: Այն տարածաշրջանում միակ վայրն է, որտեղ հնագույն ԴՆԹ-ի և սպիտակուցների պահպանման բարենպաստ պայմանները թույլ են տալիս իրականացնել մեր նախնիների և հնագույն էկոհամակարգերի գենետիկական հետազոտություններ:

Սույն աշխատանքի նպատակն է դասական մորֆոլոգիական և ժամանակակից մոլեկուլային մեթոդների կիրառմամբ վերականգնել Փոքր Կովկասի հնագույն

կենդանիների բազմազանության դինամիկայի օրինաչափությունները, և դրա հիման վրա ստուգել վերջին սառցապատման ժամանակ նշյալ տարածքը որպես ռեֆուգիալ գոտի ծառայելու վարկածը: Աշխատանքի ընթացքում ուսումնասիրվել է Քարին Տակ քարեդարյան քարանձավում հայտնաբերված ոսկրանյութը (n=835): Կիրառվել են մոլեկուլային, գենետիկական, մորֆոմետրիկ և կենսաինֆորմատիկական մի շարք մեթոդներ:

Ցույց է տրվել, որ Քարին Տակից հայտնաբերված ոսկրանյութը բնութագրվում է հնագույն ԴՆԹ-ի և սպիտակուցների պահպանվածության բարձր մակարդակով՝ բավարար հնագույն էկոհամակարգի մոլեկուլային նույնականացման և գենոմային հետազոտությունների համար: Բացի այդ, ներկայից առաջ 42-24 հազար տարով թվագրված ոսկորներից անջատված ԴՆԹ-ն առ այսօր տարածաշրջանում վերականգնված ամենահին գենետիկական նյութն է, որը բնութագրվում է կենսամոլեկուլների գերազանց պահպանվածությամբ: Առաջին անգամ Հարավային Կովկասի տարածաշրջանում Ավստրալիայի Քուրտին համալսարանի հետ համատեղ իրականացվել է հնավայրի բրածո հավաքածուի գենետիկական նույնականացում՝ ոսկորների զանգվածի մետաբարկոդավորման (bulk bone metabarcoding) եղանակով: Բրածո ոսկորների մոլեկուլագենետիկական հետազոտությունը թույլ է տվել վերականգնել ուսումնասիրված հավաքածուի 90%-ի տեսակային կազմը, մինչդեռ մորֆոլոգիապես հնարավոր է եղել նույնականացնել հավաքածուի ընդամենը 6%-ը: Դեպքերի զգալի մասում գենետիկական մոտեցումը հնարավորություն է տվել բեկորները դասակարգել մինչև տեսակի մակարդակ:

Բրածո նյութի մոլեկուլային և ֆիլոգենետիկական հետազոտության հիման վրա վերականգնվել է հնավայրի ֆաունայի հարուստ և բազմազան տեսակային կազմը՝ ներառելով 27 խումբ (կաթնասունների 11 և թռչունների 3 ընտանիք), որոնք բնակեցրել են տարածաշրջանը մոտ 42-24 հազար տարի առաջ: Վերականգնված կենդանական բազմազանությունը հիմնականում ներկայացված է ջերմասեր տաքսոններով, որոնք այսօր բնակեցնում են տարածաշրջանը, բացառությամբ վերացած չորս տեսակների: Փոքր Կովկասում քարի դարի ընթացքում ֆաունայի տաքսոնոմիական մնայուն կազմը վկայում է, որ տաք և խոնավ կլիմայով ուղեկցվող նախասառցապատումից սառը և չոր սառցապատման շրջան անցումը չի հանգեցրել տարածքի կենսաբազմազանության կտրուկ փոփոխության, ինչը հնարավորություն է տալիս տարածաշրջանը դիտարկել որպես բնական ապաստարանային գոտի, որտեղ ջերմասեր տեսակները պատսպարվել են վերջին սառցապատման ժամանակ:

АНТОНОСЯН МАРИЯ

МОЛЕКУЛЯРНЫЕ ОСНОВЫ ГИПОТЕЗЫ О МАЛОМ КАВКАЗЕ КАК РЕФУГИАЛЬНОЙ ЗОНЫ ВО ВРЕМЯ ПОСЛЕДНЕГО ОЛЕДЕНЕНИЯ

РЕЗЮМЕ

Ключевые слова: древние белки, древняя ДНК, ДНК метабаркодирование, молекулярно-генетическая реконструкция фауны.

Расположенный на географическом перекрестке между Африкой, Европой и Азией, Южный Кавказ являлся естественным коридором, через который древние люди и животные в эпоху палеолита мигрировали из Африки в Евразию. Служил ли данный регион естественным убежищем (рефугиальной зоной) для теплолюбивых видов животных и растений во время последнего оледенения? Вопрос этот остается спорным по сей день.

Недавние достижения в молекулярных технологиях привели к революционным изменениям в ряде областей науки и способствовали появлению новой дисциплины – молекулярной археологии. Сегодня детальное исследование раскопанного материала с использованием инновационных методов молекулярной биологии позволяет ответить на ключевые вопросы об особенностях древней среды обитания, что было невозможно с применением традиционных подходов.

До недавнего времени исследования подобного рода в Армении не проводились по причине того, что не удавалось выявить доисторическую стоянку с надлежащей сохранностью древних биомолекул. Обнаружение пещеры возле села Каринтак в Арцахе в 2011 году радикально изменило ситуацию, предоставив армянским биологам и археологам возможность изучения останков древних людей и животных с использованием современных молекулярных технологий.

Отложения в указанной пещере представлены непрерывной последовательностью культурных слоев, датируемых от среднего каменного века до эпохи бронзы; они содержат большое количество биологического материала человеческого и животного происхождения, семена растений и каменные орудия. Это пока единственная в регионе пещера с благоприятными условиями для сохранения древних белков и ДНК, позволяющими проводить генетические исследования древних экосистем и костных останков.

Целью данной работы является восстановление динамики разнообразия древней фауны Малого Кавказа с использованием классических морфологических и

современных молекулярных методов, а также проверка, на основе полученных данных, гипотезы о рефугиальной роли региона на протяжении последнего оледенения.

В ходе работы был изучен костный материал (n=835), обнаруженный в палеолитической пещере Карин Так. При проведении исследований использованы соответствующие молекулярные, генетические, морфометрические, морфоскопические и биоинформатические методы.

Показано, что костные останки в данной стоянке характеризуются высоким уровнем сохранности древних белков и ДНК, достаточным для геномных исследований и молекулярной реконструкции палеоэкологии. Кроме того, ДНК, выделенная из костей возрастом 24-42 тыс. лет, является самым древним в пределах Южного Кавказа идентифицированным генетическим материалом, характеризующимся превосходной сохранностью биомолекул.

Впервые, совместно с Университетом Кертина (Австралия), с использованием технологии метабаркодирования, была проведена генетическая идентификация древнего биоматериала, собранного на территории указанного региона. При молекулярно-генетическом анализе костных останков восстановлена таксономия около 90%, в то время как морфологически удалось идентифицировать лишь 6% исследуемой коллекции. В большинстве случаев генетический подход позволил классифицировать фрагменты костей до уровня вида. На основе результатов молекулярных и филогенетических исследований ископаемого материала реконструирован богатый видовой состав древней фауны, включающий 27 групп (11 семейств млекопитающих и 3 птиц), которые обитали в регионе около 42-24 тысяч лет назад. Восстановленное разнообразие животных представлено преимущественно теплолюбивыми таксонами, населяющими регион до наших дней, за исключением четырех исчезнувших видов. Устойчивость таксономической структуры фауны Малого Кавказа в рассматриваемом хронологическом интервале палеолита свидетельствует о том, что переход от жаркого и влажного межледниковья к холодно-сухому ледниковью не привел к резкому изменению биоразнообразия региона, что позволяет считать его природной зоной-убежищем, где теплолюбивые виды животных укрывались во время последнего оледенения.