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DETERMINATION OF THE PHYLOGENETIC LINEAGE OF THE SPECIES *MICROTUS ARVALIS* FROM THE REPUBLIC OF MOLDOVA BASED ON *CYTB* GENE SEQUENCING

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Abstract. Resolving phylogenetic structure in cryptic rodent species is essential for understanding evolutionary history, population connectivity, and informing conservation strategies. In the Republic of Moldova, *Microtus arvalis* co-occurs with the morphologically similar *M. levis*, complicating species-level identification based on external traits alone. To determine the phylogenetic affiliation of local *M. arvalis* populations, we sequenced the mitochondrial *cytochrome b* (*CYTB*) gene (1106 bp) from five individuals identified molecularly from a total of 13 vole samples collected in central Moldova. These sequences were analyzed alongside 26 reference sequences retrieved from GenBank, representing the major phylogenetic lineages of *M. arvalis* across Europe. Phylogenetic reconstruction, performed using the Neighbor-Joining method in R software environment, revealed that the samples from Moldova form a highly homogeneous cluster and group with sequences from Poland, Slovakia, Finland, and Hungary. This pattern indicates a clear affiliation with the eastern phylogenetic lineage. The results expand the phylogeographic understanding of *M. arvalis* in Eastern Europe and underscore the utility of mitochondrial markers in distinguishing closely related species and supporting regional biodiversity assessments.

INTRODUCTION

The *CYTB* gene of mitochondrial origin, encodes for *cytochrome b*, an integral component of the mitochondrial electron transport chain responsible for driving oxidative phosphorylation and *adenosine triphosphate* (*ATP*) production.

Its sequence exhibits low intraspecific variability and pronounced interspecific divergence, making it a reliable molecular marker for phylogenetic reconstruction. C*ytochrome b* DNA sequences from a phylogenetically and geographically diverse array of species are systematically archived in reference databases, such as GenBank and BOLD, enabling high-resolution species identification based on sequence homology, including cases where morphological traits are ambiguous due to convergence, development stage, or cryptic speciation.

Although small in size, voles exert a significant influence on the habitats they occupy, shaping trophic interactions, vegetation dynamics, and nutrient cycling. Monitoring voles populations is therefore critical, as their abundance and distribution often serve as sensitive indicators of ecosystem stability. Analysis of intraspecific genetic variation provides critical insights into the evolutionary trajectory and ecological differentiation of vole populations resolving fine-scale phylogeographic structure and demographic history. By reconstructing patterns of lineage divergence, gene flow, and population connectivity, such studies may inform the design of targeted population control strategies and resolve phylogenetic relationships among geographically or ecologically distinct populations. In the Republic of Moldova, the genus *Microtus* is represented by 2 species: *M. arvalis* (common vole) and *M. levis* (East European vole). These taxa are morphologically cryptic and often inhabit overlapping ecological niches, rendering species-level identification based solely on external characteristics unreliable during field sampling. *Microtus arvalis* is paraticularly abundant occupying open grassland habitats across a broad geographic range that extends from the Atlantic coast of Europe to Central Siberia [1]. Previous research [1] suggests that there are several phylogenetic lineages of the common vole across Europe and Asia, including western (United Kingdom, France, Spain), Italic (Italy), central (Germany, Netherlands, Denmark), eastern (Hungary, Slovakia, Poland, Ukraine, Finland) and a distinct lineage (Ukraine, Georgia, Armenia), which corresponds to the distribution of the *obscurus* form. In the present study, we sequenced and phylogenetically analyzed the mitochondrial *CYTB* gene from *M. arvalis* specimens from Moldova, with the aim of determining their lineage affiliation. The phylogenetic inference was performed through comparative analysis of *CYTB* sequences of the same species, extracted from GenBank [2,3].

MATERIALS AND METHODS

Species-level identification of Microtus arvalis and M. levis in the Republic of Moldova was not feasible during sampling due to the lack of diagnostic morphological traits and the potential for sympatric distribution across habitats. A total of 13 samples were collected, and after sequencing 5 were affiliated to *M. arvalis*. The samples were taken from the suburbs of Chisinau and from several

other localities in the central region of the country (Table 1).

Table 1. Localities and geographical coordinates where the voles included in the study were captured

Localities	**Latitude**	**Longitude**	**Identified specimens**
Piatra Alba	46.926	28.825	1 x M. arvalis
Horesti	46.806	28.929	3 x M. arvalis 3 x M. levis
Malcoci	47.016	28.665	5 x M. levis
Chisinau	47.021	28.718	1 x M. arvalis

Tissue samples were immersed in 96% ethyl alcohol for preservation and subsequently stored in a freezer until DNA extraction. DNA was extracted using the *GeneJET Genomic DNA Purification Kit* [4] using column purification technology and according to the *Thermo Fisher Scientific* manufacturer's protocol. The sequencing of the 13 samples was performed at the Institute of Ecology and Evolution of the University of Bern using the *Sanger* technology. The 5 sequences of interest with a size of 1106 bp were compared with 26 other *CYTB* sequences (extracted from *GenBank*) that represent the species *M. arvalis* and are associated with all the evolutionary lineages mentioned above. The phylogenetic analysis of 31 sequences was performed in the R programming environment [5] using the *NJ (Neighbor Joining)* method [6] the pipeline being adapted based on several sources [7, 8]. The analyzed sequences comprise all 5 mentioned phylogenetic lineages and originate from specimens from the following countries: Armenia, Denmark, Finland, France, Georgia, Germany, Hungary, Italy, Moldova, Netherlands, Poland, Slovakia, Spain, Ukraine, United Kingdom (Orkney).

RESULTS AND DISCUSSIONS

Analysis of 31 mitochondrial *CYTB* sequences revealed relevant phylogeographic structure and patterns of genetic similarity between *Microtus arvalis* specimens across different regions of Europe. The resulting genetic distance matrix delineated distinct sequence clusters, with Moldova samples exhibiting minimal pairwise divergence, indicative of high genetic homogeneity within the local population. These sequences together with sequences from Poland, Finland, Slovakia and Hungary form a well-defined cluster, suggesting a high genetic similarity between the Eastern European populations of this species (Fig. 1). Consistently low inter-sample distances, approaching zero in the heatmap, further underscore the close genetic affinity among these Eastern populations.

The presence of significant genetic homogeneity between populations from Moldova and neighboring Eastern European countries suggests a long-standing

ecological connectivity that has allowed for the maintenance of gene flow among these populations. This pattern has important implications for understanding the population dynamics of *M. arvalis* in eastern Europe and contributes to a better understanding of its phylogeographic map. The absence of major geographic barriers in this region may be a key factor in maintaining this uniform genetic structure.

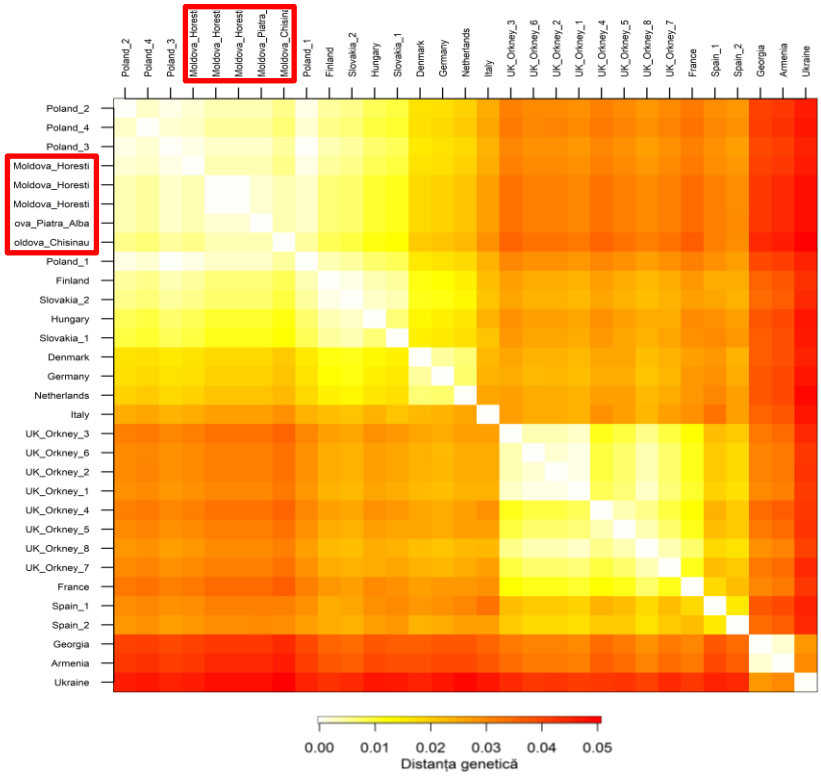


Figure 1. Genetic distance between 31 CYTB gene sequences in the species M. arvalis

Sequences from western Europe, such as those from the United Kingdom (UK), France, Spain and Italy, show some degree of genetic differentiation from eastern populations. Notably, sequences from the UK samples form a relatively homogeneous cluster, but distinct from the rest of continental Europe, confirming their geographic isolation and reinforcing the idea that oceanic barriers have a crucial role in shaping their unique genetic signature. The minimum genetic distance observed between any two sequences was 0.001 (0.1%), indicating a very close relationship, likely within the same local population or closely related individuals. The average genetic distance across all comparisons was 0.024 (2.4%), providing a measure of overall variability within the species across the studied

range. The maximum genetic distance recorded was 0.05 (5%), highlighting the most divergent evolutionary relationships found among the *M. arvalis* samples. In total, 5 clusters associated with the 5 previously described phylogenetic lineages were obtained, with the analyzed specimens from Moldova grouping with those from the Eeastern phylogenetic lineage (Fig. 2).

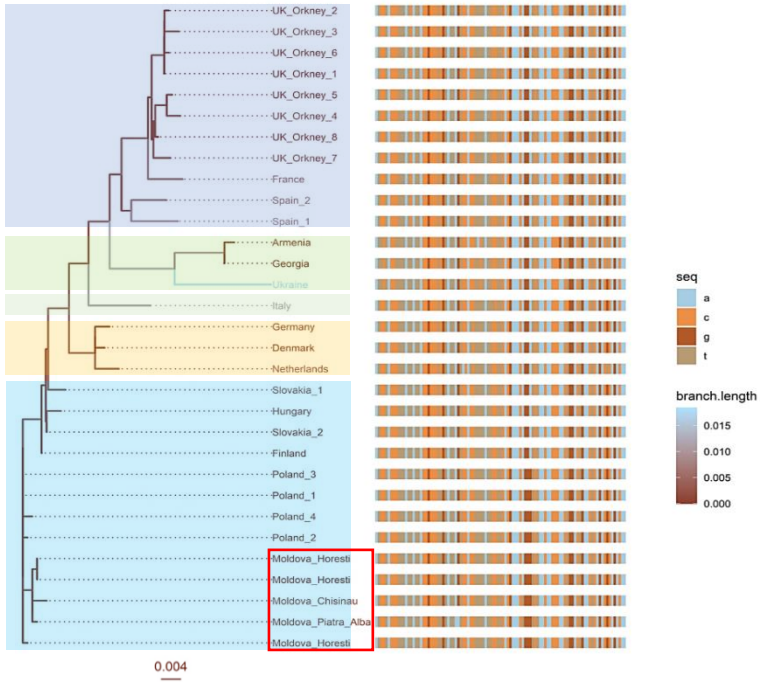


Figure 2. Phylogenetic tree obtained from sequences of the CYTB gene in the species M. arvalis

Phylogenetic lines that can be observed: 1. Western; 2. Line associated with the obscurus form; 3. Italic; 4. Central; 5. Eastern, which also includes the voles from the Republic of Moldova; a, c, g, t – adenine, cytosine, guanine, thymine.

The color scale of the tree branches represents the genetic distance and the color changes of the aligned sequences show the nucleotide differences. A relatively large difference is observed between the lineages, especially between the eastern and western clusters on the one hand and the eastern and the one associated with the *obscurus* lineage on the other. At the same time, there is no large variation between the sequences within the eastern phylogenetic lineage. The presented phylogenetic tree has 29 internal nodes and 31 top branches. The analyzed sequences have a size of 1106 bp and present 113 sites of variation. Nucleotide substitutions per site (unit of branch length) have a value of 0.004 which constitutes 0.4% genetic variation per 100 nucleotide sites. To assess the robustness of the

phylogenetic inference, we evaluated the congruence between the tree topology and the original genetic distance matrix (Fig. 3). The cophenetic correlation coefficient was 0.99, indicating an excellent fit between the tree structure and the pairwise genetic distance.

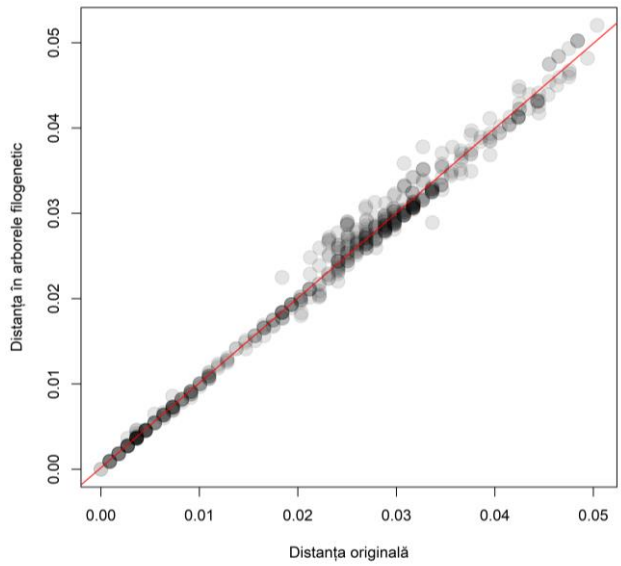


Figure 3. Correlation between the distance shown in the phylogenetic tree and the original distance

Mitochondrial DNA analysis of *M. arvalis* offers critical insights into the species evolutionary history, ecological adaptability and response to environmental change. Such data not only elucidates historical population dynamics but also informs predictive models of future evolutionary trajectories under shifting ecological conditions. These insights are essential for developing evidence-based conservation and management strategies aimed at safeguarding genetic diversity and ensuring population resilience. Furthermore, sequencing the *CYTB* gene provides valuable taxonomic resolution and enables molecular discrimination of morphologically cryptic species, such as *Microtus arvalis* and *Microtus levis*, within the Moldova fauna.

CONCLUSIONS

The study provides a phylogeographic assessment of *M. arvalis* in the Republic of Moldova based on mitochondrial *CYTB* sequences, offering a

comparative perspective within the broader European context. The samples collected in the Republic of Moldova show a high genetic homogeneity and cluster together with those from Poland, Finland, Slovakia, and Hungary. These findings confirm the affiliation of the local samples with the **eastern phylogenetic lineage** of *M. arvalis*, one of the major phylogenetic clades previously described in the literature.

All sequenced samples were registered on the global platform *Barcode of Life Data Systems* as part of the *TERVM* project – *Terrestrial Vertebrates of the Republic of Moldova* [9]. The work was conducted under the MSU subprogram no. 010701 and contract no. 01-23p- 096/03-05-2024, financed by the National Environmental Fund.

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