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Research Article
Molecular Genetic Characterization and Phylogenetic Analysis of *Pelophylax bedriagae* (Camerano, 1882) (Ranidae: *Pelophylax*)

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Abstract

Background and Objective: Molecular genetic and phylogenetic studies play an important role in clarifying the taxonomic status and evolutionary relationships of *Pelophylax bedriagae*. This study aimed to evaluate the molecular genetic characteristics and phylogenetic position of *P. bedriagae* populations from Uzbekistan based on the mitochondrial 12S rRNA gene. **Materials and Methods:** A molecular genetic approach based on the 12S rRNA gene was applied. A total of 404 base-pair sequences were analyzed, including samples collected from the Tashkent and Navoi Regions of Uzbekistan, as well as reference sequences obtained from the GenBank database. Phylogenetic analysis was conducted to determine genetic similarities and differences among taxa. **Results:** The phylogenetic analysis revealed that all analyzed taxa were grouped into 11 major clades (I-XI), which were strongly supported by high bootstrap values (88-100%). The Uzbekistan population of *P. bedriagae* clustered within Clade I, along with reference sequences from GenBank. Furthermore, the very low genetic distance values (0.08-0.32%) observed for this population indicate a high level of genetic similarity and intrapopulation stability. **Conclusion:** The obtained results confirm the high genetic similarity and taxonomic stability of *P. bedriagae* populations in Uzbekistan.

Key words: Molecular genetic, mitochondrial DNA, 12S rRNA, phylogeny, Uzbekistan

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Systematics is a fundamental scientific discipline that studies biological diversity[1]. The binomial nomenclature system, developed by Carl Linnaeus independently of evolutionary theory, laid the theoretical and practical foundation for the description and classification of biological diversity[2]. Subsequently, the approach proposed by Eviatar[3], emphasized the construction of classification systems based on phylogenetic relationships, thereby promoting a framework that reflects evolutionary relatedness. He is also recognized as one of the first scientists to develop a comprehensive phylogenetic tree model encompassing the entire animal kingdom[4].

Water frogs of the genus *Pelophylax* constitute one of the most taxonomically complex amphibian groups in the Western Palearctic. Their evolutionary history has been shaped by repeated episodes of divergence, secondary contact, introgression and hybridization, resulting in intricate phylogenetic relationships and challenging species boundaries. Consequently, the genus has become an important model system for investigating speciation processes, phylogeography and the evolutionary consequences of reticulate evolution in amphibians.

Among these taxa, *Pelophylax bedriagae* (Camerano, 1882) is widely distributed across the Eastern Mediterranean and adjacent regions of Western and Central Asia. Despite its broad distribution, the evolutionary relationships and population genetic structure of *P. bedriagae* remain incompletely understood throughout much of its range. Previous studies have largely focused on populations from Europe, Anatolia and the Mediterranean Basin, whereas populations inhabiting Central Asia remain poorly represented in molecular datasets. This geographic bias limits our understanding of the species' phylogeographic history and evolutionary diversification across its distribution range[5-7].

Molecular markers have become indispensable tools for resolving taxonomic uncertainties and reconstructing evolutionary relationships in amphibians. In particular, mitochondrial ribosomal genes have proven highly informative because of their relatively conserved structure and suitability for species-level phylogenetic analyses. The mitochondrial 12S rRNA gene is widely used in amphibian systematics and phylogenetics owing to its effectiveness in detecting genetic differentiation, assessing taxonomic status and inferring evolutionary relationships among closely related taxa. Comparative analyses of 12S rRNA sequences have contributed substantially to clarifying phylogenetic patterns within *Pelophylax* and other anuran lineages[8].

Although molecular studies have improved our understanding of water frog evolution, genetic information from Central Asian populations remains scarce. In particular, no phylogenetic assessment based on mitochondrial 12S rRNA sequences has been reported for *P. bedriagae* populations from Uzbekistan. Consequently, the phylogenetic position of these populations and their genetic affinity to other populations across the species' range remain unresolved. Addressing this gap is essential for establishing a more comprehensive picture of genetic diversity and evolutionary relationships within the species[9-11].

Therefore, the present study provides the first molecular characterization of *Pelophylax bedriagae* populations from Uzbekistan using mitochondrial 12S rRNA gene sequences and evaluates their phylogenetic relationships within the genus *Pelophylax*. By integrating newly generated sequences with publicly available datasets, this study contributes novel genetic data from a previously underrepresented region and provides insights into the evolutionary placement, genetic cohesion and taxonomic status of *P. bedriagae* in Central Asia[12,13].

The genus *Rana* is characterized among amphibians by high species diversity and a wide geographic distribution. Its representatives are distributed across almost all biogeographic regions of the Earth, except for polar areas and oceanic islands and inhabit a wide range of ecological conditions, including tundra, temperate and tropical forests, grasslands, deserts and various freshwater ecosystems[14]. Their trophic activity is primarily associated with insects and other invertebrates[15].

The ecological specialization of water frogs reflects their adaptation to different hydrological environments. For instance, *Rana lessonae* is relatively adapted to hibernating in more terrestrial conditions, whereas *Rana ridibunda* predominantly overwinters in aquatic environments[16]. These differences are associated with their morphological and physiological adaptations.

Morphometric analyses comparing samples from the type localities of *Rana bedriagae* and *Rana ridibunda* have indicated the presence of at least two morphotypes in the territory of Turkey[17]. One of these corresponds mainly to *R. bedriagae*, while the other represents a distinct morphological unit that may potentially have a separate taxonomic status.

The mitochondrial DNA of amphibians is highly conserved and possesses a compact genome structure, with gene composition and codon usage conforming to general patterns characteristic of vertebrates[18]. The mitochondrial genome typically ranges from 16 to 23 kbp in length and includes 22 tRNA genes, two rRNA genes (12S and 16S) and 13 protein-coding genes involved in energy metabolism[19].

As a result of modern molecular phylogenetic studies, water frogs have been reclassified into the genus *Pelophylax*, a proposal first suggested[20] and now widely accepted. Accordingly, the genus *Rana* has been retained mainly for terrestrial-adapted "brown frogs".

Currently, molecular genetic studies on both vertebrate and invertebrate animals are being actively conducted in our country. In particular, molecular genetic research on invertebrates[21-25] fishes[26] and lizards[27,28] is being carried out.

The present study aims to characterize, for the first time in the Republic, the species *Pelophylax bedriagae* (Camerano, 1882), belonging to the genus *Pelophylax*, based on the mitochondrial 12S rRNA gene from samples collected in the Tashkent and Navoi Regions of Uzbekistan.

MATERIALS AND METHODS

Ethical approval: This study was conducted in full compliance with international bioethical standards and accepted guidelines for the care and use of animals, involving specimens of *Pelophylax bedriagae*. The research protocol was reviewed and approved by the Ethics Committee of the Zoologists Society under the Institute of Zoology, Academy of Sciences of the Republic of Uzbekistan (Approval No. 0121: 12.03.2026).

All experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals. During sample collection and research, all necessary measures were taken to minimize stress to the animals and to avoid any harm to their natural habitats.

No ethical standards were violated in the use of animals during the course of this study.

Genetic sampling: In this study, genetic samples of *Pelophylax bedriagae* belonging to the genus *Pelophylax* were collected during 2025-2026 in the Tashkent (40°55'20.22"N, 65°57'59.61"E) and Navoi (41°34'23.89"N, 69°46'55.91"E) regions of the Republic of Uzbekistan. Tissue samples were taken from the toe (foot) region and preserved in 96% ethanol solution (Fig. 1).

Genomic DNA extraction and Polymerase Chain Reaction (PCR): DNA was extracted from the selected samples using the GeneJET Genomic DNA purification kit[29-31].

Genomic DNA extracted from *P. bedriagae* was used to determine the nucleotide sequences of the 12S rRNA gene. For this purpose, the universal mitochondrial 12Sa primer (5-AACTGGGATTAGATACCCCACTAT-3) and reverse primer 12Sb (5-TGAGGAGGGTGACGGGCGGT-3). The PCR amplification was carried out under the following conditions:

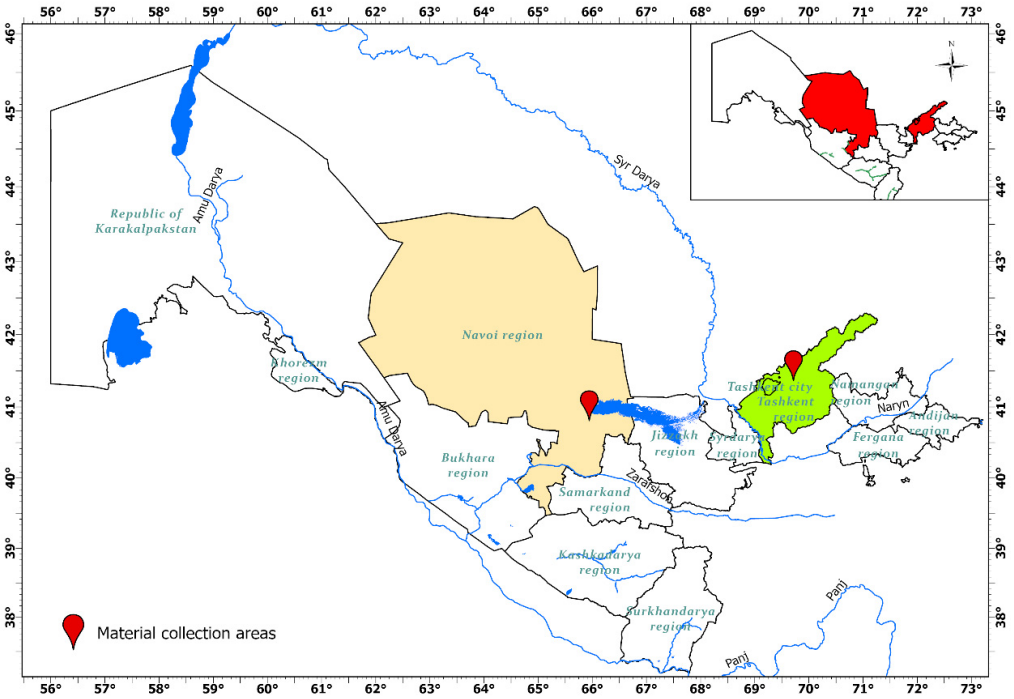


Fig. 1: Sampling locations of *Pelophylax bedriagae* (Camerano, 1882) belonging to the genus *Pelophylax* from which genetic samples were collected

An initial denaturation at 93°C for 1 min, followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min[32,33].

The presence of DNA in the PCR products was confirmed by electrophoresis on a 1.0% agarose gel at 120 V. DNA amplification and gel purification were carried out according to the manufacturer's instructions using reagent kits provided by "Silex M" (Moscow, Russia). DNA sequencing was performed using the ABI PRISM® BigDye™ Terminator v. 3.1 Cycle Sequencing Kit and sequencing reactions were analyzed on an ABI PRISM 3100-Avant Genetic Analyzer (Moscow, Russia).

Phylogenetic tree construction: For phylogenetic analysis, 12S rRNA gene nucleotide sequences from 31 specimens representing 13 species of the genus *Pelophylax.* As outgroups, sequences of *Hylarana macrodactyla* with GenBank accession number LC061604 were included.

The obtained nucleotide sequences were manually edited using BioEdit version 7.0.5.2[34] and subsequently all sequences were aligned using the MAFFT algorithm[35]. Phylogenetic trees were reconstructed using the Maximum Likelihood (ML) method implemented in IQ-TREE 2[36]. The best-fitting evolutionary model was selected using the ModelFinder algorithm, which is integrated into the IQ-TREE software[37].

The reliability of the tree topology was assessed using 1000 bootstrap replications[38]. Additional analyses were performed using Bayesian Inference (BI) with the MrBayes 3.1.2 software[39]. The MCMC (Markov Chain Monte Carlo) algorithm was run for 4 million generations, with samples taken every 1000 generations. The first 1000 trees were discarded as burn-in. Phylogenetic tree reliability was evaluated using Bayesian posterior probabilities (PP) and bootstrap values (BS) as follows: PP $\geq$0.95 and BS $\geq$70%-high support; PP = 0.90-0.94 and BS = 50-69%-moderate support; PP<0.90 and BS<50%-low support[40]. The final phylogenetic trees were visualized graphically using the iTOL (Interactive Tree of Life) online platform[41].

RESULTS AND DISCUSSION

As a result of the conducted molecular genetic analyses, a nucleotide sequence comprising 404 base pairs of the mitochondrial 12S rRNA gene of *Pelophylax bedriagae*, belonging to the genus *Pelophylax*, was identified.

Based on the results of the study, the phylogenetic relationships among populations of *P. bedriagae* and closely related representatives of the genus *Pelophylax* (synonym: *Rana*) were investigated using nucleotide sequences of the mitochondrial DNA 12S rRNA gene.

For the analysis, nucleotide sequences obtained from the GenBank database, as well as a *P. bedriagae* sample collected from Uzbekistan (Tashkent and Navoi Regions), were used. The phylogenetic tree was constructed using *Hylarana macrodactyla* as an outgroup.

The phylogenetic tree was divided into several well-defined clades (I-XI), all of which were strongly supported by high bootstrap values (88-100%). In particular, Clade I (blue), located in the upper part of the phylogenetic tree, included the *P. bedriagae* sample from Uzbekistan together with *P. bedriagae* sequences from GenBank (NC029200, NC029201) and *R. bedriagae* specimens (AB640931, AB640920, AB640901), forming a single monophyletic cluster. The genetic distance within this cluster ranged from 0.0008 to 0.0032 (0.08-0.32%). This clade was strongly supported by a high bootstrap value (approximately 96%), indicating a high level of genetic similarity among the samples.

Clade II (green) included *P. kurtmuelleri* and *R. kurtmuelleri* sequences (AJ222653, AJ222655, MT410949, NC026895). The genetic distance within this clade ranged from 0 to 0.0008 (0-0.08%), indicating very low intraspecific variation. This clade was strongly supported by a bootstrap value of 91%, demonstrating a high level of reliability.

Clade III (purple) comprised *R. ridibunda* sequences (JN627421, AB640897). The genetic distance within this group was 0.0008 (0.08%), indicating low internal divergence. This clade was supported by a bootstrap value of 90%.

Clade IV (yellow) consisted of *R. caralitana* sequences, with genetic distances ranging from 0.0156 to 0.0188 (1.56-1.88%). This clade was supported by a bootstrap value of 88% and showed relatively higher genetic divergence compared with the other clades.

Clade V (blue-green) included *P. lessonae* and *R. lessonae* sequences, with genetic distances ranging from 0 to 0.0028 (0-0.28%), indicating high genetic similarity. This clade was supported by bootstrap values of 93-95%.

Clade VI (dark orange) consisted of *R. bergeri* sequences, with genetic distances ranging from 0.0015 to 0.0113 (0.15-1.13%). This clade was supported by an approximate bootstrap value of 92%.

Clade VII (purple) included *P. plancyi* sequences, with genetic distances ranging from 0 to 0.028 (0-2.8%). Despite the relatively higher internal variation, this clade was strongly supported by a bootstrap value of 100%, indicating high reliability.

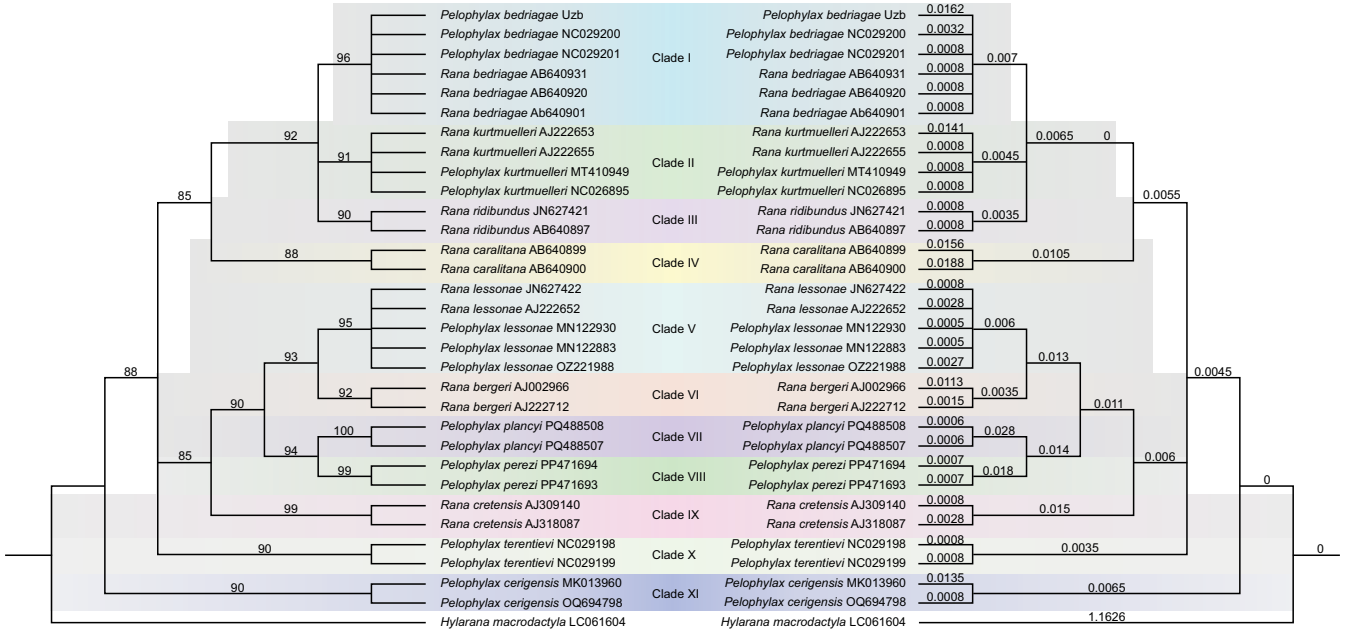


Fig. 2: Phylogenetic tree of representatives of the genus *Pelophylax* constructed based on nucleotide sequences of the mitochondrial DNA 12S rRNA gene

Clade VIII (green) consisted of *P. perezi* sequences, with a genetic distance of 0.0007 (0.07%). This clade was strongly supported by a bootstrap value of 99%.

Clade IX (pink) included *R. cretensis* sequences, with genetic distances ranging from 0.0028 to 0.015 (0.28-1.5%). This clade was strongly supported by a bootstrap value of 99%.

Clade X (light green) comprised *P. terentievi* sequences, with genetic distances ranging from 0 to 0.0035 (0-0.35%). This clade was supported by an approximate bootstrap value of 90%.

Clade XI (blue) consisted of *P. cerigensis* sequences, with genetic distances ranging from 0.0065 to 0.0135 (0.65-1.35%). This clade was also supported by an approximate bootstrap value of 90% (Fig. 2).

Overall, the obtained results demonstrated that all clades in the phylogenetic tree were strongly supported by high bootstrap values (88-100%). The separation of Clade I indicated that the Uzbekistan population of *P. bedriagae* has a very close genetic relationship with other geographic samples, with low genetic distances (0.08-0.32%). At the same time, relatively higher genetic divergence observed in some clades (particularly Clades IV and VII) suggests the presence of ongoing evolutionary differentiation among populations. Such genetic differentiation is likely to have been shaped by geographic isolation, ecological factors and natural selection.

As a result of the conducted molecular genetic studies, the nucleotide sequences of the mitochondrial DNA 12S rRNA gene of *P. bedriagae* were deposited in an international bioinformatics database (ncbi.nlm.nih.gov) and the corresponding accession numbers were obtained (*P. bedriagae* Tashkent-PZ365371; Navoi-PZ344945).

Water frogs belonging to the genus *Pelophylax* represent one of the most extensively studied taxonomic groups among amphibians distributed across Europe and Asia[42].

At the same time, most studies on this group have primarily focused on their unusual reproductive mechanism based on hybridogenesis. However, information on their genetic diversity and broad-scale evolutionary history remains geographically limited, scattered in the literature, or not fully systematized.

This situation is particularly relevant for endemic *Pelophylax* taxa of the Balkan Peninsula, which have so far been only partially studied in terms of comprehensive range-wide phylogeographic analyses[41-43].

The clustering of the Uzbekistan population of *P. bedriagae* together with GenBank reference sequences within Clade I indicates a high level of genetic stability at the species level, as well as a strong genetic affinity with geographically distant populations. The very low genetic distances observed within this clade (0.08-0.32%) suggest the absence of significant genetic divergence among populations.

The level of genetic differentiation observed in other clades indicates that evolutionary relationships among species are at different stages of divergence. In particular, the relatively high genetic divergence detected in Clades IV and VII suggests that evolutionary separation processes are actively ongoing within these taxa. This pattern may be associated with factors such as geographic isolation, differences in ecological conditions and natural selection pressure.

The obtained results demonstrate that the interspecific genetic structure within the genus *Pelophylax* is complex, with strong genetic similarity preserved among some taxa, while others exhibit pronounced divergence. This, in turn, confirms that evolutionary processes within this group are still actively ongoing.

CONCLUSION

In summary, phylogenetic analyses of species belonging to the genus *Pelophylax* based on the mitochondrial 12S rRNA gene revealed a well-structured and highly supported clade topology within the group. All major clades were strongly supported by high bootstrap values (88-100%), indicating that the reconstructed phylogeny is stable and reliable.

In particular, the clustering of *P. bedriagae* populations, including the Uzbekistan sample, with GenBank reference sequences within a single clade, as well as the very low genetic distances (0.08-0.32%), confirms a high level of genetic similarity and consolidation within this species. This suggests that, despite geographic separation, these populations form a genetically stable group.

At the same time, the relatively high genetic divergence observed among certain taxa, particularly in Clades IV and VII, indicates that evolutionary divergence processes are still ongoing within these lineages. This pattern is likely associated with geographic isolation, differences in ecological conditions and natural selection pressure.

SIGNIFICANCE STATEMENT

This study demonstrates the high accuracy and reliability of a molecular phylogenetic approach based on the mitochondrial 12S rRNA gene for resolving phylogenetic relationships and assessing genetic diversification within the genus *Pelophylax*. The obtained results confirm a high level of genetic consolidation of *P. bedriagae* populations distributed in Uzbekistan with the global genetic pool, as well as their low intra-taxon divergence. This research further substantiates the importance of molecular markers in evaluating amphibian evolution, phylogeography and taxonomic stability.

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