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Phylogenetic Relationships Characterization of *Bufotes pewzowi* (Bedriaga, 1898) Inhabiting near Aquatic Basins of Central and South Uzbekistan

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ABSTRACT

This study analyzed partial nucleotide sequences of the mitochondrial DNA (mtDNA) 16S rRNA gene region in *Bufotes pewzowi* (Bedriaga, 1898). Over 580 nucleotides were amplified using PCR and subsequently sequenced. The resulting sequence data were compared with entries in the NCBI database and showed a 99.83% match with *B. pewzowi*. During the study, the nucleotide sequence of the mtDNA 16S rRNA gene for the *B. pewzowi_Uzb* specimen was submitted to NCBI and assigned the accession number PV124861. Molecular analysis confirmed that this specimen is a distinct species within the family Bufonidae, identified as *B. pewzowi*. Future research will enhance understanding of this species' morphological traits and contribute to amphibian taxonomy. Phylogenetic analysis revealed that species within the Bufonidae family form four monophyletic groups, with bootstrap support values ranging from 50% to 100%. The monophyletic group containing the genus *Bufotes*, including *B. pewzowi_Uzb*, showed bootstrap support values between 75% and 100%, confirming its placement within the phylogenetic tree.

INTRODUCTION

Currently, the number of scientific research works dedicated to molecular biology (genomics) in various groups of organisms is continuously expanding. The methods of morphological, physiological, biochemical, anatomical, ethological, ecological, zoogeographical, and molecular-genetic research are the foundation of modern biological sciences regarding species identification in the animal kingdom. Morphological research methods are still widely used in the systematics of animals. However, sometimes the morphological method does not assist in identifying closely related species or "polymorphs." In recent years, the significant advancement of biochemical and

molecular-genetic research and the introduction of new investigation methods have opened up the field of molecular taxonomy.

Amphibians participate in the stability of the food chain as an important part of the ecosystem. The subtropical and arid climate conditions of Uzbekistan provide a favorable environment for aquatic animals, including amphibians. For amphibians, the water bodies of the region (lakes, rivers, swamps, and irrigation systems in rural areas) are significant factors, including water temperature, mineralization levels, and plant cover. Depending on the geographical location of the water bodies, temperature changes, decreases in water levels, or pollution are observed, and the density and distribution dynamics of species can also vary **(Stöck, *et al.,* 2001; Litvinchuk *et al.,* 2011, 2012; Martin *et al.,* 2017; Showler, 2018; Dufresnes *et al.,* 2019; Shernazarov & Jumayev, 2021)**. At the same time, amphibians participate as intermediate, reservoir, and primary hosts in the biological cycles of numerous helminths, consistently exerting their influence on the epizootiological and epidemiological status of the environment **(Ikromov *et al.,* 2023; Aliyev *et al.,* 2024)**.

Currently, molecular genetic studies are being used in the taxonomic and systematic classification of the fauna of Uzbekistan, including studies on nematodes of the digestive system of ruminants **(Kuchboev *et al.,* 2020; Turgunov *et al*., 2024)**, freshwater invertebrates **(Madumarov *et al.,* 2021)**, parasitic nematodes in fish **(Soatov *et al*., 2023; Bazarbayeva *et al*., 2024)**, phytonematodes in plants **(Mirzaev *et al*., 2024)**, and on freshwater vertebrates **(Nomonov *et al*., 2024a)**.

Scientific research on the molecular-genetic identification of the *Bufotes pewzowi* (Bedriaga, 1898) species has expanded globally in recent years. **Vences and Holman, (2008)** conducted a molecular-genetic analysis of amphibians in the genus *Bufo*, providing important information on the molecular classification of *Bufotes pewzowi* and its relations with similar species. In the study by Ruchin and colleagues, the phylogenetic relationships of *Bufotes pewzowi* and other amphibian species in the Caucasus were identified using molecular genetic methods, expanding knowledge about the evolutionary history and phylogeny of the genus *Bufo* **(Ruchin & Shchinov, 2013)**. **Zhang and Li (2015)** conducted interspecies phylogenetic analysis based on the mitochondrial genome of amphibians in the genus *Bufo*. They attempted to determine various approaches and the ecological role of *Bufotes pewzowi* using mitochondrial DNA. **Fouquet *et al.* (2012)** analyzed the molecular phylogenetic relationships among amphibians in the *Bufo* group, discussing the phylogenetic position of *Bufotes pewzowi* and its connections with other European amphibians.

According to **Litvinchuk *et al.* (2010, 2011, 2012)**, comprehensive multilocus genomic analyses (RAD-seq) on the phylogeography of *Bufotes* in Palearctic green frogs have been conducted, including mitochondrial modeling and phenotypic data (bioacoustics, morphometry, toxin composition), studied and analyzed. Due to hybridization among frogs in the Bufonidae family, the genotype of *Bufotes pewzowi*

(Bedriaga, 1898) has become tetraploid, originating from *B. latastii* and *B. perrini* species **(Stöck *et al.*, 2001, 2006, 2013; Ficetola & Stöck, 2016)**. In Uzbekistan, *Bufotes pewzowi* (Bedriaga, 1898) has previously been referred to as the green frog *Bufo viridis* (Laurenti, 1768) **(Showler, 2018; Dufresnes *et al.*, 2019)**. These analyses indicate that research on the molecular genetic identification of *Bufotes pewzowi* is crucial for determining the species' phylogenetic placement and genetic diversity. Future genome- wide analyses and ecological monitoring studies would allow for a deeper understanding of the species.

The current study aimed to analyze the molecular-phylogenetic relationships of the *Bufotes pewzowi* (Bedriaga, 1898) species belonging to the *Bufotes* genus of amphibians in Central and South Uzbekistan.

MATERIALS AND METHODS

Collection of genetic material

During the years 2024-2025, scientific materials were collected using directional and stationary research methods from various geographical areas of the southern and central regions of our Republic at the following coordinates: Kashkadarya region 39°0'51.73"N, 65°23'43.94"E 38°58'1.82"N, 65°16'29.85"E; 39°13'55.88"N, 65°7'54.75"E; Surxondaryo region 37°46'58.91"N, 67°16'49.60"E 37°25'46.65"N, 66°58'34.13"E; Bukhara region 39°49'55.48"N, 64°41'41.30"E; 39°34'2.03"N, 64°42'9.91"E (Fig. 1).

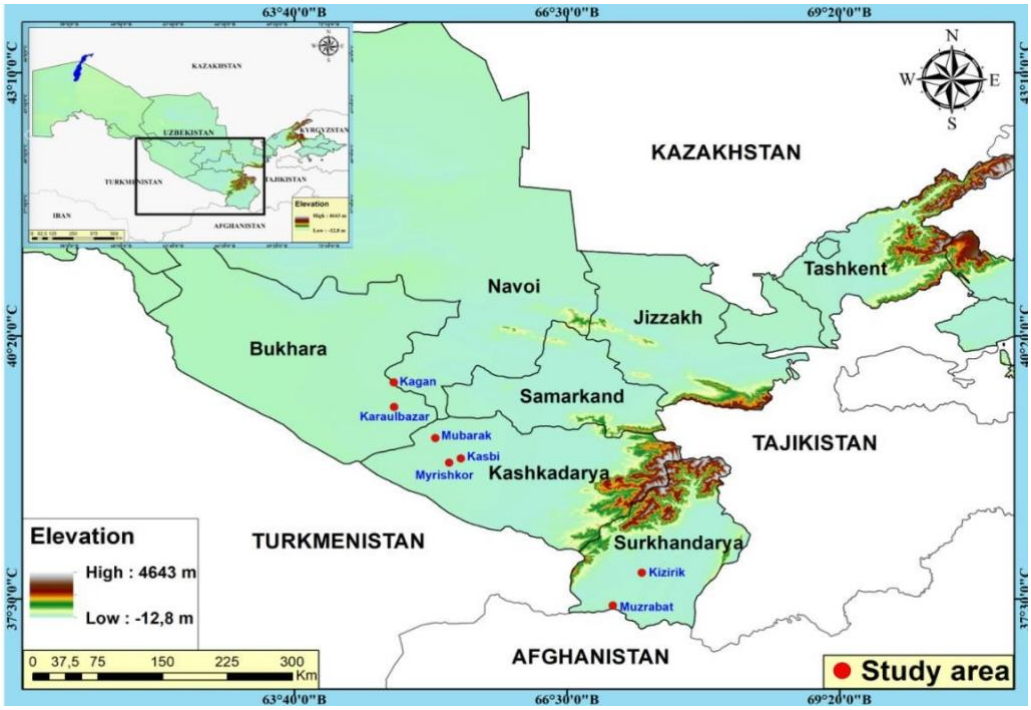

Fig. 1. Map of the geoinformation system of points for collecting amphibian samples

The identification of amphibian species composition was carried out with the scientific staff of the Zoology Institute's Laboratory for the Accounting and Cadastre of Rare Animal Species. At the same time, research on the species composition of Central Asian amphibians utilized studies by foreign **(Clark, 1990; Stöck *et al.,* 2001; Dujsebayeva, 2008, 2010; Litvinchuk *et al.,* 2011; Fouquette & Dubois, 2014; Wagner *et al.,* 2016; Martin *et al.,* 2017; Showler, 2018; Dufresnes *et al.,* 2019)** and local **(Siddikov *et al.,* 1998; Vashetko & Siddikov, 1999; Shernazarov & Jumayev, 2021)** scientists, as well as international scientific websites (AMNH; AmphibiaWeb; Gbif).

Molecular genetic method

Samples preserved in 70% ethanol were used to extract genomic DNA using the DNeasy Blood and Tissue Kits for DNA Isolation (Qiagen Inc., November 2023). The concentration of each DNA sample was determined using a spectrophotometer (Thermo Fisher Scientific, China). The extracted genomic DNA samples are stored at –20°C until used for DNA polymerase chain reaction (PCR).

PCR recipe

For the polymerase chain reaction (PCR), primers that read the nucleotides belonging to the *16S rRNA* region of mitochondrial DNA (*mtDNA*), widely used for molecular-genetic identification of amphibians, were utilized **(Hebert *et al.,* 2003a, 2003b)**. The PCR mixture was prepared with 26.4µl of bidistilled water, 4µl of 10x Taq buffer, 0.8µl of dNTP, 2µl of each primer (16S cp-F CGAGGGCTTTACTGTCTCTT and 16S cp-R CCTATTGTCGATATGGACTCT), 4 µl of DNA template, and 0.8 µl of Taq polymerase, totaling 40µl. The PCR was carried out in the following steps: 3 minutes at 92°C, followed by 35 cycles of 15 seconds at 92°C, 30 seconds at 55°C, 30 seconds at 72°C, and a final step of 10 minutes at 72°C **(Vences & Holman, 2008)**.

The presence of DNA in PCR products was determined by electrophoresis on a 1.0% agarose gel with a voltage of 100V. During DNA amplification and extraction from the gel, the manufacturer's instructions were followed using a set of reagents produced by "Sileks M" (Moscow, Russia).

DNA sequencing was carried out using the ABI PRISM® BigDye™ Terminator v. 3.1 reagent kit, and the reaction products were sequenced at GATC Biotech AG. The analysis of the obtained nucleotide sequences was conducted using specialized computer programs such as Bioedit, ClustalX2, DNAstarTM, and PAUP4 **(Hall, 1999; Larkin *et al.,* 2007)**.

Constructing a phylogenetic tree

To construct a phylogenetic tree, sequences of amphibian nucleotides obtained from sequencing and DNA sequences from the International Biotechnology Information Center database (https://www.ncbi.nlm.nih.gov) were used, and these sequences were manually edited using the Genious Prime software. Consensus sequences were calculated using the Mega X Computational software. Initial data obtained from this program and

additional sequences from the NCBI database were compared using the MAFFT v.7 online program **(Katoh *et al.*, 2005)** and the Clustal Omega 1.2.2 program with standard settings and edited in the Geneious Prime software. The obtained *mtDNA 16S rRNA* region nucleotide sequences were identified through maximum likelihood (ML) phylogenetic tree analysis with 100 repetitions performed via ultra-fast loading in the IQ- TREE version 1.6.12 **(Trifinopoulos *et al.*, 2016)**, and analyses were conducted on the CIPRES Science Gateway V 3.3. To facilitate the production of consensus trees of *mtDNA 16S rRNA* region nucleotide sequences of the Bufonidae family species, *Anaxyrus boreas* (FJ882830) was included as an outgroup. The resulting phylogenetic tree was analyzed and edited in the iTOL v6.6 software (https://itol.embl.de/login.cgi) **(Letunic & Bork, 2021)**.

For the phylogenetic analysis of *Bufotes pewzowi* (Bedriaga, 1898), *mtDNA* gene sequences from 30 samples belonging to the genera *Bufotes, Strauchbufo, Duttaphrynus,* and *Anaxyrus* were used from the data of the International Biotechnology Information Center (NCBI) (Table 1).

Table 1. Information about the species of the genus *Bufotes* from the NCBI database

S. No.	Amphibian species	Input code ID	Country
1	*Anaxyrus boreas*	FJ882830	Belgium
2	*Strauchbufo raddei*	GU183855	Belgium
3	*Strauchbufo raddei*	LC640543	Japan
4	*Strauchbufo raddei*	OP056178	China
5	*Duttaphrynus stomaticus*	PP851073	India
6	*Duttaphrynus stomaticus*	MT983004	Nepal
7	*Duttaphrynus stomaticus*	MT982996	Nepal
8	*Duttaphrynus melanostictus*	MT982983	Nepal
9	*Bufo cf. boulengeri*	EU497457	Italy
10	*Bufo danatensis*	AF160796	China
11	*Bufo danatensis*	AF171211	China
12	*Bufotes sp. Kandahar*	MG700149	Afghanistan
13	*Bufotes sp. Kandahar*	MG699933	Afghanistan
14	*Bufo siculus*	EU497443	Italy
15	*Bufo siculus*	EU497627	Italy
16	*Bufo balearicus*	EU497630	Italy
17	*Bufotes oblongus*	KT031467	USA
18	*Bufotes cf. pewzowi*	FJ882811	Belgium
19	*Bufotes_pewzowi*	PV124861	Uzbekistan
20	*Bufotes zamdaensis*	NC046047	China
21	*Bufo baturae*	FJ861308	Pakistan
22	*Bufotes pseudoraddei*	KT031479	USA

23	*Bufotes pseudoraddei*	MW133482	USA
24	*Bufotes turanensis*	NC062077	Tajikistan
25	*Bufotes shaartusiensis*	KP739220	Tajikistan
26	*Bufotes variabilis*	NC050665	USA
27	*Bufotes sitibundus*	KT031492	USA
28	*Bufotes sitibundus*	KT031495	USA
29	*Bufo viridis*	AY862557	Turkey
30	*Bufotes cf. luristanicus*	GU226835	Belgium

RESULTS

Molecular-genetic identification

Molecular phylogenetic studies were conducted on 78 specimens of the genus *Bufotes* caught in 2024-2025 from the regions of Southern and Central Uzbekistan.

Currently, the study of the *16S rRNA* region of *mtDNA* allows for the identification of amphibian species with close molecular phylogenetic relationships. To accurately identify amphibians and to resolve certain unresolved taxonomic issues, molecular-genetic methods are widely used alongside morphological methods to identify controversial species and phenotypic variations. According to the results of molecular- genetic research, 580 nucleotide pairs belonging to the *16S rRNA* region of *mtDNA* of the species *B. pewzowi* were isolated, and to compare these species, the following were used from NCBI (https://blast.ncbi.nlm.nih.gov): *B. pewzowi_Uzb* (accession number: PV124861), *B. pewzowi* (accession number: FJ882811), and *B. viridis* (accession number: AY862557).

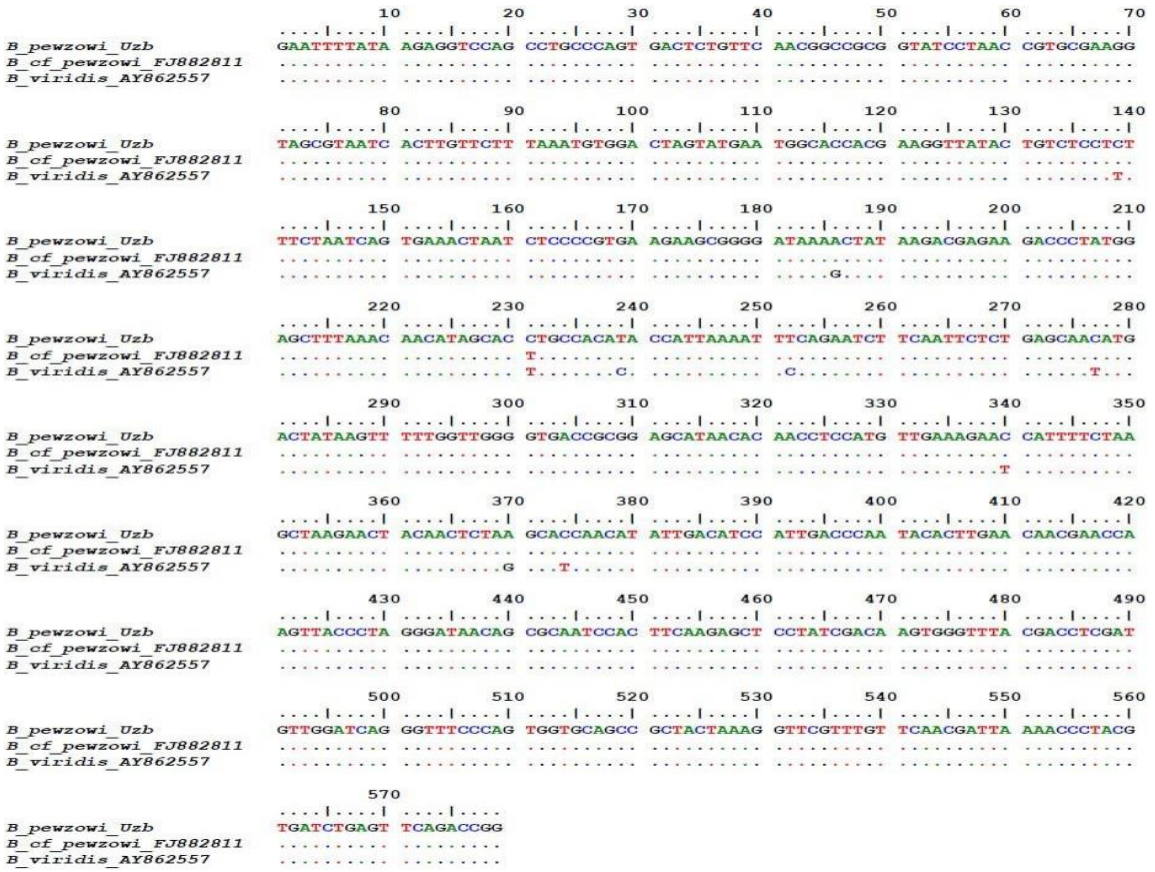

Fig. 2. Nucleotide sequence of the *16S rRNA* region of *mtDNA* of species belonging to the genus *Bufotes*

It is evident that among the samples collected from the southern regions of our republic, there is a single nucleotide difference between the *Bufotes pewzowi_Uzb* sample and the nucleotide sequence of *B. pewzowi* obtained from the NCBI database. This difference occurs at the 231st nucleotide, where the *B. pewzowi_Uzb* sample contains C-cytosine, while the NCBI database sample has T-thymine. The similarity between the nucleotides in the *mtDNA 16S rRNA* region is 99.83% (Fig. 2).

There are 9 nucleotide differences between the *Bufotes pewzowi_Uzb* sample and the nucleotide sequence of *B. viridis* obtained from the NCBI database. These differences occur at nucleotides 139, 231, 276, 340, and 374, where the *B. pewzowi_Uzb* sample contains C-cytosine, while the NCBI database sample has T-thymine in the *B. viridis* sample. At nucleotides 186 and 370, the *B. pewzowi_Uzb* sample contains A-adenine, while the NCBI database sample has G-guanine in the *B. viridis* sample. At nucleotides 239 and 252, the *B. pewzowi_Uzb* sample contains T-thymine, while the NCBI database sample has C-cytosine in the *B. viridis* species. The similarity between the nucleotides in the *mtDNA 16S rRNA* region is 98.5% (Fig. 2).

Phylogenetic tree

Bootstrap values given at the nodes of a phylogenetic tree indicate the level of confidence. Values such as 100, 87.5, 75, 62.5, and 50 are present, with higher values indicating strong confirmation of relationships between species. The scale of the phylogenetic tree represents genetic differences or the number of mutations. The distances between species reflect how far they have diverged from their common ancestor.

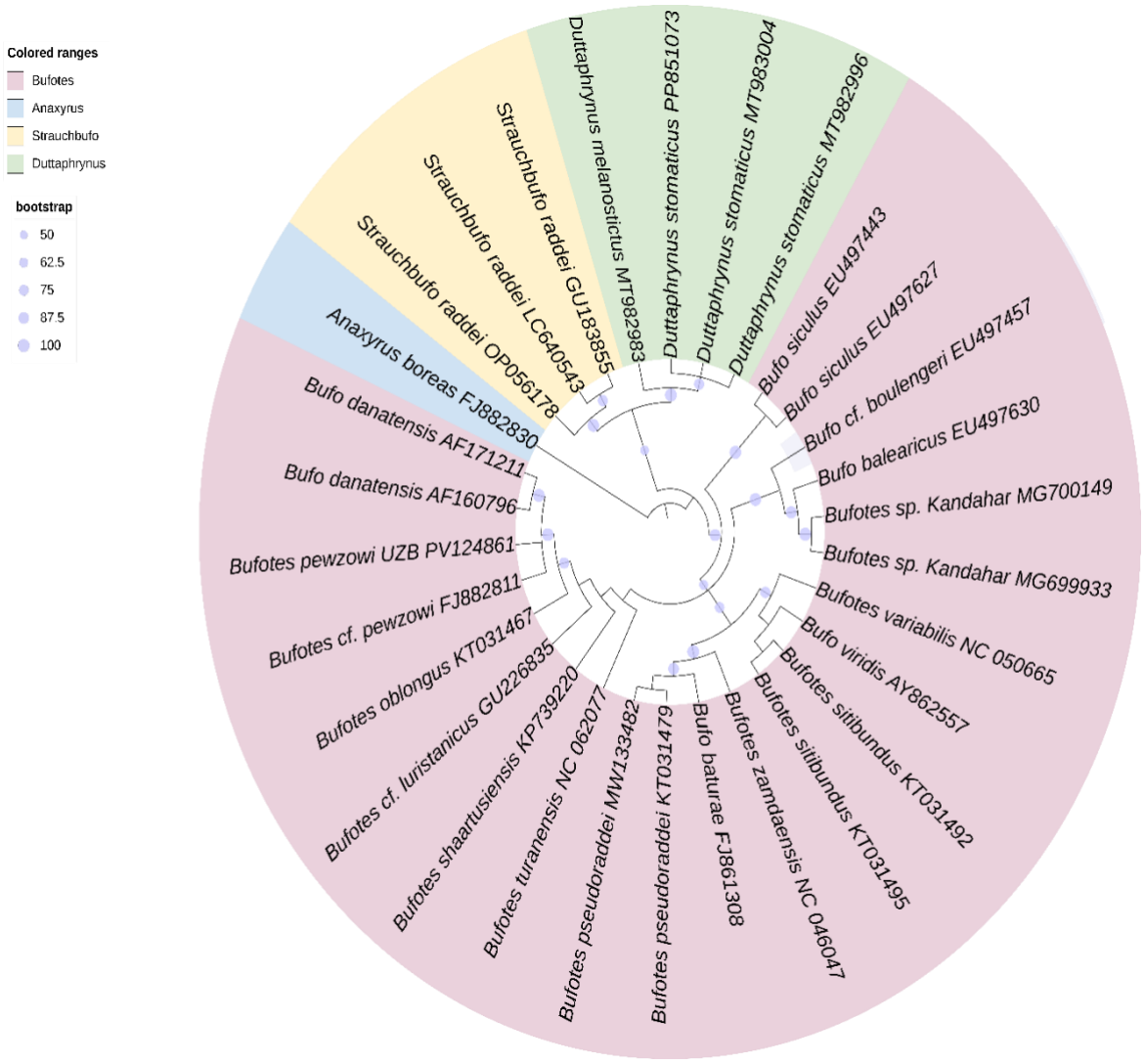

Fig. 3. Phylogenetic tree of species belonging to the family Bufonidae based on the ML method

This phylogenetic tree shows the evolutionary relationships among species in the Bufonidae family: *Bufotes, Strauchbufo, Duttaphrynus,* and *Anaxyrus*. The tree was rooted, and *Anaxyrus boreas* (FJ882830) was isolated as the most distant relative (outgroup). According to the study's results, it was found that amphibians are grouped

into four main clades (monophyletic groups) based on the analysis of nucleotide sequences from the 16S rRNA domain of the obtained mitochondrial DNA (mtDNA) and nucleotide sequences retrieved from the NCBI database (Fig. 3).

Anaxyrus (outgroup) – This clade contains only one specimen of *Anaxyrus boreas* (FJ582830) and is not closely related to other species.

Strauchbufo (yellow) - This clade consists of specimens of *Strauchbufo raddei* (GU183855, LC640543, OP056178), forming a robust monophyletic group. The internal nodes of this clade are joined with 100% bootstrap support. It is also moderately related to the *Duttaphrynus* clade, with 87.5% bootstrap support.

Duttaphrynus (orange) – This clade includes *Duttaphrynus stomaticus* (MT983004, PP851073, MT982996) and *D. melanostictus* (MT982983). The internal relationships of this genus are very strong, with 100% bootstrap support at the corresponding nodes. This situation indicates that the genus *Duttaphrynus* is a genetically coherent and separated group.

Bufotes (pink) - The largest clade and contains many species, including *Bufotes pewzowi, Bufotes viridis, Bufotes sitibundus, Bufotes variabilis, Bufotes pseudoraddei, Bufotes balearicus,* and other *Bufo* and *Bufotes cf.* specimens. Within this clade, the relatedness between species is very close, with some internal nodes merging with 100% bootstrap support, while others merge with 75–87.5% bootstrap support. This indicates a high level of diversification and stable genetic relationships within the clade.

CONCLUSIONS AND RECOMMENDATIONS

Until now, *B. pewzowi* has been frequently recorded in Uzbekistan based on its morphological characteristics. This study is the first molecular-genetic characterization of *B. pewzowi* in Uzbekistan. *B. pewzowi_Uzb* sample with *B. pewzowi* sample obtained from the NCBI database was 99.83% similar to the nucleotides of the 1*6S rRNA* region of *mtDNA*.

When the phylogenetic relationships were studied, it was found that representatives of the Bufonidae family formed 4 monophyletic groups in the phylogenetic family tree and produced a bootstrap loading value of 50-100%. The low value of bootstrap loading observed in some monophyletic groups indicates that the species in this monophyletic group are close to the evolutionary separation period.

*Bufotes pewzowi*_uzb is located in the central part of the Bufotes clade, and its genetic proximity to other local species (such as *Bufotes viridis* and *Bufotes variabilis*) has been confirmed with high bootstrap support (87.5%). This analysis helped clarify the taxonomic structure of the Bufonidae family and provided a deeper understanding of their evolutionary history, creating an important foundation for future ecological and genetic studies.

The results of the molecular phylogenetic analysis of the *B. pewzowi* species confirmed that it is an independent species. In the future, the genomic research of this species will help study the phylogeny and ecological adaptations of the species in depth. This study shed light on the molecular identification and classification of *B. pewzowi* species, and showed the advantage of the PCR-based sequence method for determining its ecological and biogeographical place, and species composition.

GRATITUDE

We express our gratitude to the scientific team of the laboratory "Registration and Cadastre of Rare Animal Species" at the Institute of Zoology of the Academy of Sciences of the Republic of Uzbekistan for their practical assistance in identifying the composition of amphibian species, and to the leadership of the scientific project "Molecular Genetic Classification of Wild Vertebrate Species of Bukhara and Navoi Regions" for conducting the molecular genetic analysis.

NOVELTY STATEMENT

Molecular phylogenetic identification of the *Bufotes pewzowi* amphibian was carried out for the first time in Uzbekistan. According to the results of molecular genetic studies, the sequence of 580 pairs of nucleotides belonging to the *16S rRNA* domain of *mtDNA* of the *Bufotes pewzowi* species was extracted and deposited in the National Center for Biotechnology Information (NCBI) and received accession number (PV124861).

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