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Molecular genetic characterization and phylogenetic placement of *Teratoscincus rustamovi* (Squamata, Sphaerodactylidae)

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This study examines the molecular-genetic and phylogenetic status of *Teratoscincus rustamovi*, a gecko species endemic to the sandy deserts of southwestern Uzbekistan. Formerly considered a subspecies of *T. scincus*, it has recently been recognized as a distinct species, yet molecular evidence has been limited. We analyzed mitochondrial 16S rRNA sequences obtained from new samples and compared them with congeneric taxa using Maximum Likelihood phylogenetic reconstruction, pairwise genetic distances, and Automatic Barcode Gap Discovery (ABGD). Results revealed that *T. rustamovi* forms a strongly supported monophyletic clade (100% bootstrap) and shows clear divergence (0.07–0.12) from *T. keyserlingii*, *T. scincus*, *T. roborowskii*, and *T. przewalskii*. Heatmap visualization and ABGD consistently confirmed its recognition as an independent molecular unit. Importantly, the sequence of *T. rustamovi* was submitted for the first time to international databases. These findings validate the taxonomic status of *T. rustamovi* as a separate evolutionary lineage and highlight the role of mitochondrial markers in resolving reptile phylogenetics. Given its restricted distribution and vulnerability to habitat change, the results also provide a molecular basis for conservation strategies in Central Asian desert ecosystems.

Keywords: *Teratoscincus rustamovi*; molecular phylogeny; 16S rRNA; species delimitation; genetic distance; ABGD; Sphaerodactylidae; Central Asia; endemic species; conservation genetics.

Introduction

In recent decades, molecular-genetic approaches have become fundamental in modern herpetology, providing robust tools for species delimitation, taxonomy, and conservation biology (Kluge, 1993). Globally, reptile diversity has been extensively reassessed using molecular phylogenetics, revealing numerous cryptic species and reshaping the systematics of geckos and related groups (Böhm et al., 2013). Within the order Squamata, the family Sphaerodactylidae represents one of the most diverse lizard lineages, distributed across both the New and Old World, with particularly high adaptive radiation in arid environments (Shcherbak & Golubev, 1996; Gamble et al., 2008). Despite this, phylogenetic relationships within the family remain partially unresolved, as earlier classifications, based primarily on morphological traits, have sometimes conflicted with genetic evidence (Kluge, 1995).

The genus *Teratoscincus* Strauch, 1863, commonly known as "frog-eyed geckos," is an ecologically specialized group inhabiting arid and semi-arid landscapes of Central Asia and the Middle East (Szczerbak, 1979; Nazarov et al., 2017). These lizards are characterized by large eyes, granular scales, and distinctive plate-like caudal morphology, which confer adaptations to sandy desert habitats. Taxonomic research on *Teratoscincus* has a long history, with several revisions based on morphology, distribution, and ecology (Szczerbak, 1979; Shcherbak & Golubev, 1996). However, the discovery of *T. rustamovi* in the Kyzylkum Desert of Uzbekistan by Nazarov & Rajabizadeh (2019) represented a significant milestone, as it highlighted the persistence of unrecognized endemism in one of Central Asia's least studied desert ecosystems. Earlier, this taxon had been described as a subspecies (*T. scincus rustamovi*) by Szczerbak (1979), but molecular data later supported its recognition as a full species.

Current evidence suggests that *T. rustamovi* possesses a restricted distribution and distinct ecological adaptations within sandy habitats of Uzbekistan, resembling other desert-dwelling reptiles that show strong microhabitat specialization (Bondarenko, 2020). Importantly, the conservation status of desert reptiles in Central Asia remains under-investigated, despite mounting threats from climate change, overgrazing, and anthropogenic disturbance (Stuart et al., 2004; Böhm et al., 2013). As a narrowly distributed endemic, *T. rustamovi* represents both a phylogenetically and ecologically vulnerable lineage that requires focused study.

Up to the present, animals of the fauna of our republic, including insects (Kadirov et al., 2024; Kimyonazarov et al., 2024), nematodes (Aliyev, 2024; Mirzaev, 2024), and fish (Quvatov et al., 2023; Ubaydullayev et al., 2025), have been studied at the molecular level.

Mitochondrial DNA markers, particularly the 16S rRNA gene, have proven highly informative in reptile phylogenetics, enabling the resolution of interspecific relationships and species boundaries (Han et al., 2004). Within *Teratoscincus*, however, discrepancies between morphological systematics and molecular evidence remain (Kluge, 1995), underscoring the need for targeted phylogenetic analyses. Studies in other regions, such as Central Iran, have already demonstrated the presence of cryptic diversity within *Teratoscincus* (Nazarov et al., 2017), raising questions about evolutionary differentiation within desert habitats.

Given these gaps, the present study focuses on elucidating the molecular-genetic and phylogenetic characteristics of *T. rustamovi* using the mitochondrial 16S rRNA marker. The objectives are (1) to clarify its phylogenetic position within Sphaerodactylidae, (2) to assess its genetic distinctiveness relative to congeners (*T. keyserlingii*, *T. scincus*, *T. roborowskii*, *T. przewalskii*), and (3) to provide baseline molecular data essential for conservation. By integrating phylogenetic and distance-based analyses, this work contributes to a broader understanding of reptile diversification in Central Asian deserts, while also offering valuable insights for species conservation strategies (Pianka & Vitt, 2003; Losos, 2011).

Materials and methods

DNA samples were obtained from the collection of the Institute of Zoology, Academy of Sciences of the Republic of Uzbekistan, and the study organism, *Teratoscincus rustamovi*, is depicted in its natural habitat (Fig. 1).

Fig 1. *Teratoscincus rustamovi* in its natural habitat (living in a sandy environment)

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, November 2023). Before extraction, biological materials had been preserved in 70% ethanol. DNA concentrations were measured using a Thermo Fisher Scientific spectrophotometer (China). The isolated DNA was stored at –20°C for future use in polymerase chain reaction (PCR) assays. To amplify the mitochondrial 16S rRNA gene – a widely used marker in amphibian molecular identification – specific primers were applied, as described by Hebert et al. (2003a, 2003b). The PCR reaction volume was 40 µL, comprising 26.4 µL of double-distilled water, 4 µL of 10× Taq buffer, 0.8 µL of dNTPs, 2 µL each of the forward (16S cp-F: CGAGGGCTTTACT GTCTCTT) and reverse (16S cp-R: CCTATTGTCGATATGGAC TCT) primers, 4 µL of template DNA, and 0.8 µL of Taq DNA polymerase. Amplification conditions included an initial denaturation at 92 °C for 3 minutes, followed by 35 cycles of denaturation at 92 °C for 15 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final elongation step was performed at 72 °C for 10 minutes. The success of PCR amplification was verified by electrophoresis on a 1.0% agarose gel at 100 V. Resulting DNA bands were excised and purified using a commercial gel extraction kit (Sileks M, Moscow, Russia), following the manufacturer's protocol. Sequencing was conducted using the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit, with processing carried out by GATC Biotech AG. Nucleotide sequences obtained were analyzed using a combination of software tools, including BioEdit, ClustalX2, DNAstar™, and PAUP4 (Hall, 1999; Larkin et al., 2007).

To determine the molecular phylogenetic relationships of *Teratoscincus rustamowi*, the 16S rRNA gene sequences were analyzed alongside those of other *Teratoscincus* species retrieved from the GenBank database. A representative sequence from a different genus was included as an outgroup. Sequence alignments were conducted using the MAFFT algorithm (Katoh et al., 2002) and manually refined with BioEdit version 7.0.5.2 to ensure alignment quality. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method implemented in the IQ-TREE 2 software package (Minh et al., 2020). The best-fitting nucleotide substitution model was automatically selected using the ModelFinder module. Node support was assessed via 1,000 ultrafast bootstrap replicates (Felsenstein, 1985). The final phylogenetic tree was visualized using the Interactive Tree of Life (iTOL) online tool (Letunic & Bork, 2021), which enabled an interactive and customizable representation of phylogenetic relationships.

To evaluate intra- and interspecific genetic divergence based on the 16S rRNA gene sequences, genetic distance analysis was performed using the Automatic Barcode Gap Discovery (ABGD) algorithm (Puillandre et al., 2012). This method facilitates the identification of molecular operational taxonomic units (MOTUs) and the automatic delimitation of species boundaries. Initially, pairwise genetic distances were calculated using the Kimura 2-parameter (K2P) model in MEGA X software (Kumar et al., 2018). The resulting distance matrix was then analyzed using the ABGD web platform (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). The following parameters were used: minimum intraspecific divergence P_{min} = 0.001, maximum intraspecific divergence P_{max} = 0.1, relative gap width (X) = 1.5, recursive partitioning = enabled, and the distance model = K2P. The barcode gap detected by the algorithm allowed for the delineation of distinct molecular units and provided insights into the number of putative species. This molecular approach added robustness to species identification and complemented traditional morphological classification.

Results

To clarify the evolutionary relationships among species and genera within the family Sphaerodactylidae, a phylogenetic tree was constructed using mitochondrial DNA sequences and the Maximum Likelihood (ML) method (Fig. 2). *Aristelliger expectatus* was selected as the outgroup to root the tree, providing a reliable external reference for interpreting the phylogenetic relationships among the ingroup taxa. The bootstrap (BS) values shown at the nodes represent the statistical support for each inferred relationship.

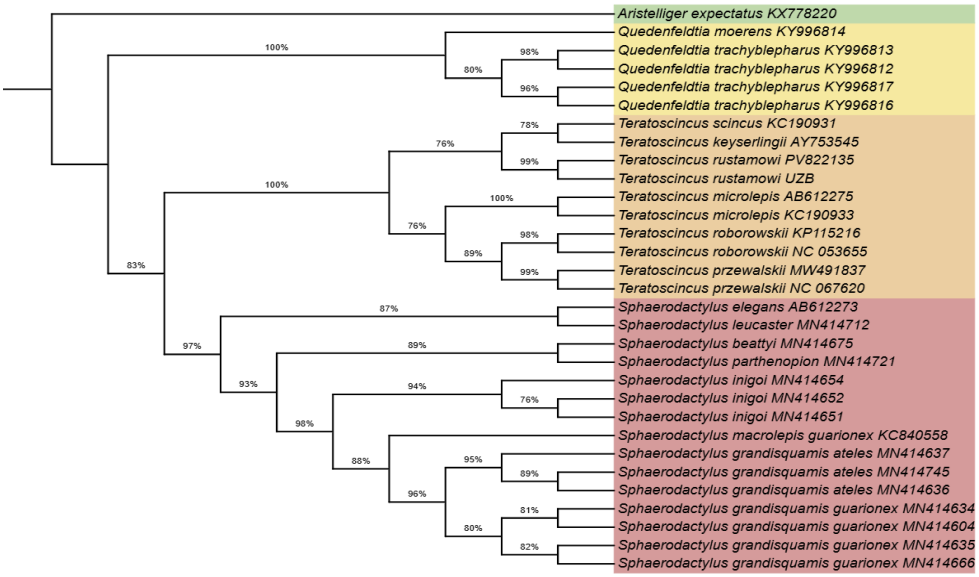

Fig. 2. Phylogenetic relationships among *Teratoscincus rustamovi* and other representatives of the family Sphaerodactylidae based on nucleotide sequences of the 16S gene: the tree was constructed using the Maximum Likelihood (ML) method, with the numbers above the nodes indicating bootstrap (BS) values

The phylogenetic tree revealed five distinct monophyletic clusters:

– *Quedenfeldtia* cluster: this group includes multiple specimens of *Q. moerens* and *Q. trachyblepharus*; the high bootstrap values ranging from 80% to 98% indicate strong genetic similarity among these taxa;

– *Teratoscincus* cluster: this cluster comprises *T. rustamovi*, *T. scincus*, *T. keyserlingii*, *T. microlepis*, *T. roborowskii*, and *T. przewalskii*; notably, the *T. rustamovi* sample from Uzbekistan and the GenBank sequence PV822135 formed a strongly supported subgroup with a 100% bootstrap value, indicating complete genetic concordance; furthermore, *T. roborowskii* and *T. przewalskii* showed close phylogenetic affinity, supported by a 99% bootstrap value;

– *Sphaerodactylus elegans* cluster: this group contains *S. elegans* and *S. leucaster*, whose close relationship is supported by a bootstrap value of 87%;

– *Sphaerodactylus inigoi* cluster: this cluster includes *S. beattyi*, *S. parthenopion*, and *S. inigoi*; their internal relationships are supported by BS values ranging from 88% to 94%, suggesting strong genetic relatedness;

– *S. grandisquamis* and *S. macrolepis* cluster: this group comprises *S. macrolepis* and several subspecific forms of *S. grandisquamis* (*ateles, guanicae,* and *guanireno*); bootstrap values within this group range from 76% to 96%, reflecting regional diversification and genetic differentiation among subspecies.

Overall, the high bootstrap values observed across major nodes of the tree (76–100%) confirm the robustness of the inferred phylogenetic relationships. In particular, the consistent clustering of *T. rustamovi* samples suggests strong genetic cohesion within the species and supports its status as a distinct and stable taxonomic entity.

A heatmap based on pairwise genetic distances calculated from mitochondrial DNA sequences was used to visually represent the levels of genetic similarity and divergence among the species included in this study (Fig. 3). In the heatmap, darker colors indicate greater genetic similarity (i.e., smaller distances), while lighter colors reflect greater divergence. Key observations among species of the genus *Teratoscincus* are as follows: The most closely related species are *T. przewalskii* (NC_067620 and MW491837) and *T. roborowskii* (NC_053655 and KP115216), with almost negligible genetic distances (0.0000010 and 0.0019225, respectively), indicating a high level of phylogenetic relatedness. Similarly, low divergence (0.0222812) was observed between *T. microlepis* sequences (KC190933 and AB612275), suggesting genetic stability within this species. The main focus of this study, the *T. rustamovi* specimen, shows genetic distances of 0.1072743 and 0.0770278 from *T. keyserlingii* (AY753545) and *T. scincus* (KC190931), respectively.

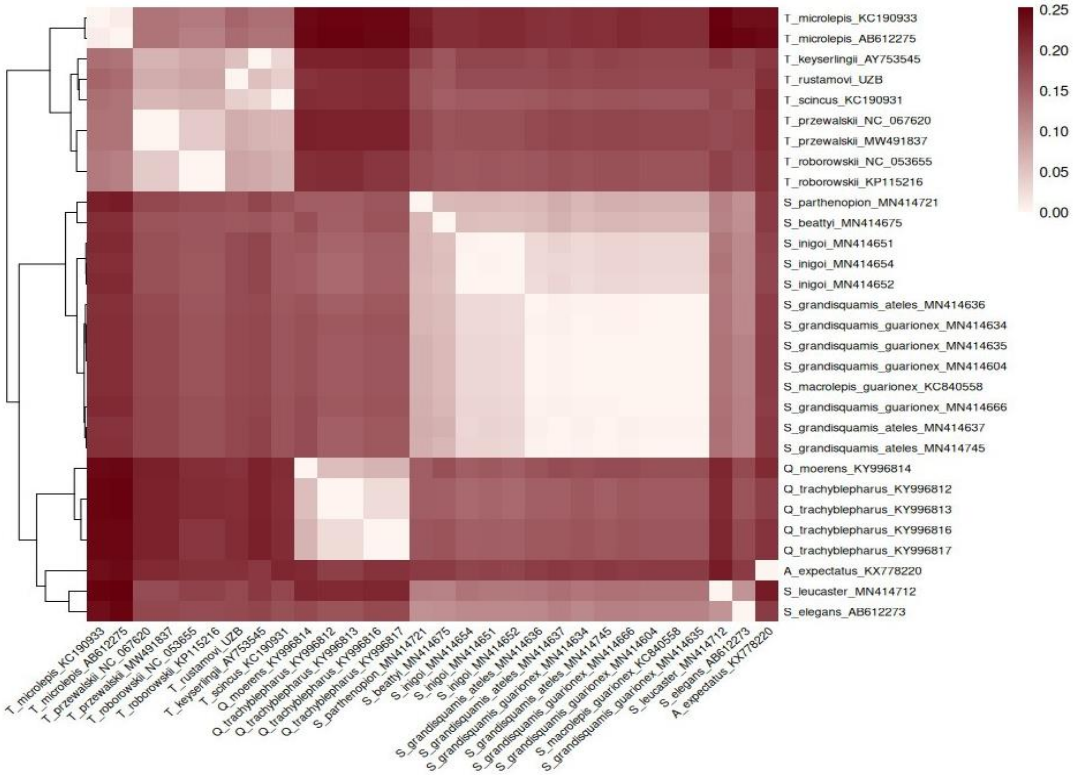

Fig. 3. This heatmap illustrates the genetic distances based on the 16S gene between *T. rustamovi* and other congeneric species: the differences in genetic distances between the samples are represented by color variations which aid in identifying phylogenetic relationships among the species

Despite phylogenetic proximity, these values indicate that *T. rustamovi* stands out as a distinct genetic entity. These results support the notion that *T. rustamovi* has well-defined taxonomic boundaries and can be considered a separate species. Within the genus *Quedenfeldtia*, almost no genetic divergence (0.0000010) was found among *Q. trachyblepharus* sequences (KY996812–KY996817), indicating strong intra-species genetic homogeneity. In contrast, a genetic distance of 0.0666357 was observed between *Q. moerens* (KY996814) and *Q. trachyblepharus*, highlighting a clear interspecific distinction. Species within the genus *Sphaerodactylus* also exhibited varying levels of phylogenetic relatedness. The genetic divergence among *S. inigoi* (MN414651, MN414652, MN414654) and *S. grandisquamis* (MN414604, MN414635, and others) ranged from 0.0021 to 0.0155, indicating a high level of relatedness. *S. macrolepis guarionex* (KC840558) was also closely positioned within this group. Conversely, *S. leucaster* (MN414712) displayed genetic distances ranging from 0.1749 to 0.2045 relative to other *Sphaerodactylus* species, reflecting its distant evolutionary placement.

Overall, the genetic distances observed through the heatmap corroborate the phylogenetic relationships inferred from the tree. In particular, the distinct and stable grouping of *T. rustamovi* as a genetically independent entity provides strong support for its recognition as a valid taxon.

Genetic distances between *T. rustamovi* and related species were assessed using the ABGD (Automatic Barcode Gap Discovery) approach based on 16S gene sequences. The histogram (Fig. 4) revealed a clear "barcode gap" separating intraspecific distances (0.00–0.05) from interspecific ones (>0.10), supporting the recognition of *T. rustamovi* as a distinct species. The ranked distance plot (Fig. 4) showed a gradual increase from 0.03 to 0.26, highlighting the genetic

divergence of the examined specimens from other taxa. Across a range of prior intraspecific divergence values (P = 0.0017–0.0567), the ABGD analysis identified 8–10 clusters, with recursive partitioning refining group boundaries.

According to the distance matrix (Fig. 4), genetic distances within *T. rustamovi* were approximately 0.07–0.12, while divergence from *T. microlepis* averaged ~0.16 and from *T. przewalskii* exceeded 0.11. Distances to *Aristelliger* and *Sphaerodactylus* lineages were above 0.20, indicating more distant phylogenetic relationships.

Overall, both ABGD clustering and genetic distance analyses consistently indicate that *T. rustamovi* represents a genetically distinct and independently evolving species.

Discussion

The results of the phylogenetic analysis demonstrated clear evolutionary divergence within the genus *Teratoscincus*. This indicates that *T. rustamovi* is also molecularly distinct from other species in the genus. *T. rustamovi* was first described as a new species in 2009, with its range restricted to the southern regions of Uzbekistan – specifically the desert-mountain areas of Surkhandarya and adjacent territories (Nazarov et al., 2010). Despite its morphological similarities to species like *T. scincus* and *T. keyserlingii*, phylogenetic distances based on molecular markers have revealed significant differences (Macey et al., 1999, 2000). These distinctions provide strong evidence supporting the taxonomic status of *T. rustamovi*. Moreover, other species within the genus *Teratoscincus* have also formed several independent clusters, indicating a degree of genetic diversification within the group (Zheng & Wiens, 2016). The high genetic affinity observed between *T. microlepis*, *T. roborowskii*, and *T. przewalskii* (BS: 99%) confirms their phylogenetic closeness, yet the fact that each species is positioned within its distinct cluster suggests that they have followed independent evolutionary trajectories. The inclusion of *T. rustamovi* samples collected from Uzbekistan adds valuable new data. This not only broadens the known distribution range of the species but also provides a foundation for studying its genetic diversity. Thus, the results of this analysis further confirm the taxonomic validity of *T. rustamovi* as an endemic species on a molecular level.

Molecular phylogenetic analyses revealed significant genetic divergence between *T. rustamovi* and other species in the genus. In particular, the genetic distance between *T. rustamovi* and *T. keyserlingii* (AY753545) was found to be 0.0608, indicating considerable phylogenetic separation. This value is relatively high compared to other pairwise distances within the genus and supports the idea that *T. rustamovi* has evolved as an independent evolutionary lineage. The heatmap visualization further confirmed these genetic distinctions, showing that *T. rustamovi* also exhibits considerable genetic differences from *T. microlepis* (0.057), *T. przewalskii* (0.070), and *T. roborowskii* (0.068). These findings suggest that *T. rustamovi* occupies a distinct phylogenetic position on the molecular level. Given that the samples of *T. rustamovi* were collected only from the southwestern regions of Uzbekistan (specifically, Bukhara and Kashkadarya Provinces), this species' genetic isolation from other *Teratoscincus* species is likely due to geographic isolation. Such separation may have led to genetic divergence through the accumulation of unique mutations, ultimately resulting in the species' distinctiveness. Therefore, *T. rustamovi* should be considered a separate phylogenetic unit. The genetic distance matrix also shows that *T. rustamovi* is markedly divergent from species like *T. keyserlingii* (0.1073) and *T. scincus* (0.0770), reinforcing its genetic consistency and evolutionary stability. These findings support the recognition of *T. rustamovi* as a genetically distinct taxonomic unit. In contrast, within the genus *Quedenfeldtia*, the genetic similarity among *Q. trachyblepharus* sequences (genetic distance: 0.0000010) indicates high genetic cohesion, while the distance to *Q. moerens* (0.0666) confirms a clear interspecific divergence. Similarly, in the genus *Sphaerodactylus*, significant phylogenetic differences were observed. For instance, *S. leucaster* exhibited genetic distances ranging from 0.1749 to 0.2045 from other congeners, indicating its development along an independent evolutionary pathway.

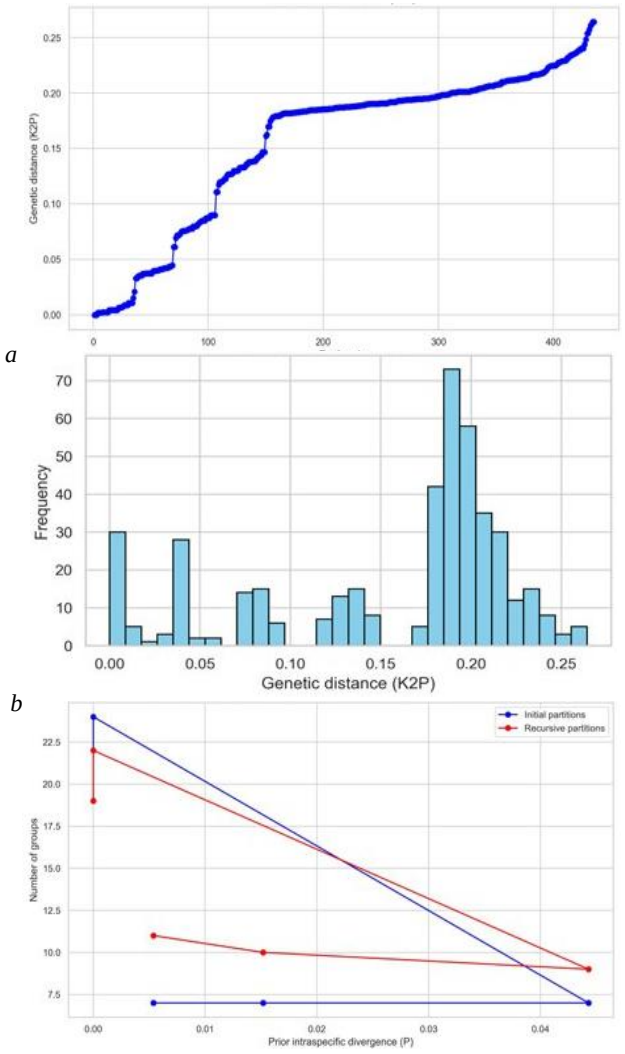

Fig. 4. Results of Automatic Barcode Gap Discovery (ABGD) analysis: *a* – sorted genetic distances (X – rank order, Y – genetic distance, K2P); *b* – histogram of genetic distances (X – genetic distance, K2P; Y – frequency); *c* – prior intraspecific divergence graph (X – prior intraspecific divergence, P; Y – number of groups; initial and recursive partitions shown in different colors

Overall, the heatmap generated from mitochondrial DNA-based genetic distances provides strong support for the relationships identified in the phylogenetic tree. The genetic distinctiveness and stability of *T. rustamovi* as an independent and cohesive group strongly suggest the need to re-evaluate its taxonomic status and recognize it as a separate species.

This study employed analyses based on the Automatic Barcode Gap Discovery (ABGD) algorithm to delineate the genetic boundaries of *T. rustamovi*. The genetic distance distribution derived from the 16S rRNA gene revealed a distinct barcode gap (≈0.05), effectively separating intraspecific (0.00–0.05) from interspecific (≥0.10) divergence. These findings confirm that even morphologically similar species can be distinguished through molecular markers (Fujita et al., 2012).

The examined genetic distances ranged from 0.03 to 0.26, indicating substantial genetic variability among samples. The prior intraspecific divergence plot from the ABGD analysis identified 8–10 genetic clusters, while recursive partitioning confirmed the stability of these groupings. This supports the recognition of *T. rustamovi* as a distinct species from *T. microlepis* and *T. przewalskii* (Hebert et al., 2003; Puillandre et al., 2012).

Larger genetic distances (>0.20) further clarify the phylogenetic position of this species within the family Sphaerodactylidae. The results of molecular delimitation reaffirm that species-level differentiation can be reliably detected even when morphological differences are

minimal. This approach not only strengthens the taxonomic status of *T. rustamovi* but also provides an important scientific basis for uncovering hidden diversity within the herpetofauna of Central Asia.

Conclusion

This study provides new molecular evidence supporting the taxonomic status of *T. rustamovi* as a distinct and evolutionarily independent species within the genus *Teratoscincus*. Phylogenetic analysis based on mitochondrial 16S rRNA gene sequences revealed clear genetic differentiation between *T. rustamovi* and other congeneric species. The observed genetic distances, particularly the significant divergence from *T. keyserlingii*, *T. microlepis*, *T. przewalskii*, and *T. roborowskii*, highlight *T. rustamovi*'s unique phylogenetic position.

Visualizations such as heatmaps, genetic distance matrices, and ABGD-based species delimitation further substantiated these findings. The presence of a distinct "barcode gap" and multiple clusters in the ABGD analysis confirms that *T. rustamovi* possesses sufficient genetic divergence to be considered a valid species. The geographic isolation of *T. rustamovi* populations in Southwestern Uzbekistan – particularly in Bukhara and Kashkadarya provinces – likely contributed to its genetic differentiation through limited gene flow and accumulated mutations.

Altogether, these results reinforce the taxonomic validity of *T. rustamovi* and emphasize the importance of using integrative approaches, including molecular tools, for accurately identifying and delineating species, especially in morphologically similar taxa. This work also contributes valuable data for future conservation efforts and biogeographic studies of Central Asian geckos.

The results of the molecular-genetic research and the data obtained from bioinformatic programs led to the first-ever submission of the nucleotide sequence of *T. rustamowi* to an international bioinformatics database, where it was assigned an accession number (Accession Number: *Teratoscincus rustamowi* – PV822135).

This research work was carried out within the framework of the research program of the Institute of Zoology of the Academy of Sciences of the Republic of Uzbekistan for 2025–2029, "1.2. Creation of a digital information system of the animal world of the Bukhara and Navoi regions", financed from the state budget.

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