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The First Molecular Characterization of Baltistan or Karakorum Gecko, *Altiphylax stoliczkai* (Steindachner, 1867) from Leh, Ladakh, India

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Author's contribution

Author AB conceived and designed the manuscript. Analysis and interpretation of the data, the drafting of the paper, and revising it critically for content done by author AB.

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ABSTRACT

The distribution of saurian species in Ladakh is less documented compared to mammals and birds. Additionally, there are very few molecular studies on the lizard species inhabiting the high altitudes of this region. In this study, we collected two road-killed specimens of *Altiphylax stoliczkai* (Steindachner, 1867), commonly known as the Baltistan gecko or Karakorum gecko, from Ganglaas, Leh, Ladakh. This locality is newly recorded for the species. We conducted a molecular analysis based on the COX1 gene to differentiate between *Altiphylax stoliczkai* and *Altiphylax tokobajevi* (Jeremcenko & Szczerbak, 1984). This research represents the first molecular analysis of *Altiphylax stoliczkai* from Leh, Ladakh, India.

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Keywords: Sauria; Altiphylax; COX1; Ladakh.

1. INTRODUCTION

Recent reports indicate that one in five reptilian species faces the threat of extinction, with an additional one in five classified as Data Deficient (Bohm et al., 2013). A global decline in reptile populations has been attributed to various factors, including habitat fragmentation, climate change, illegal and pet trade, invasive species, pollution, and disease. Urgent action is required to implement effective conservation plans for these species. Primack (2010) emphasized the necessity of understanding the distribution and data availability for reptile conservation (Bahuguna et al., Cox et al., 2009, Gibbons et al., 2000). Palearctic naked-toed geckos, which include angular-toed geckos, comprise a diverse group of 101 species within the Gekkonidae family, distributed from North Africa across southwestern and Central Asia to northern India, western China, and southern Mongolia. Notably, these geckos lack adhesive subdigital pads. The group encompasses taxa assigned to several genera, including *Agamura, Alsophylax, Altigekko, Altiphylax, Asiocolotes, Bunopus*, and others. For example, *Altiphylax tokobajevi* is found in Kyrgyzstan, preferring shrub land habitats at altitudes of 1,800–2,500 meters (5,900–8,200 feet) (Khan et al., 2010). The genus *Altiphylax* includes five species: *Altiphylax baturensis*, *Altiphylax levitoni, Altiphylax mintoni, Altiphylax stoliczkai*, and *Altiphylax tokobajevi*.

Key distinguishing features among groups are slightly bent-toed, weakly tuberculate geckos with long, thin limbs and tail (*Agamura, Rhinogecko*); straight-toed, atuberculate geckos (*Asiocolotes, Microgecko, Tropiocolotes*); straight- to slightly bent-toed, variably tuberculate geckos with enlarged nasal scales (*Alsophylax, Bunopus*); straight- to slightly bent-toed, weakly tuberculate geckos with scales forming lateral fringes on toes (*Crossobamon, Pseudoceramodactylus, Stenodactylus*); distinctly bent-toed, variably (often heavily) tuberculate geckos (*Carinatogecko, Cyrtodactylus, Cyrtopodion*, *Indogekko, Mediodactylus, Siwaligekko, Tenuidactylus*) are the main features that apply to distinguish various groups.

The taxonomic and nomenclatural history of these geckos has been reviewed by many researchers including Szczerbak and Golubev (1986, 1996) (25,26), Krysko *et al.* (2007). Further work by Khan, (1998, 1991, 1993a, 1993b, 1990) and others resulted in the discovery of many new bent-toed gecko species from northern Pakistan and adjacent regions. *Altiphylax stoliczkai* (Steindachner) was described from a single specimen collected by Ferdinand Stoliczka in 1865 near Karoo, north of Dras, in northern Kashmir (Blanford, 1878). This single specimen was transferred to the Naturhistorisches Museum, Wien (Vienna, Austria), where Steindachner designated it as the holotype (NMW 16756) in honor of its collector. The holotype is well-illustrated by Szczerbak and Golubev (1986, 1996). Stoliczka also collected an additional 46 specimens from the type locality during the Second Yarkand Expedition (1873-1874), and a few localities eastward to Leh in the Indus River valley of central Ladakh, Kashmir. These specimens were subsequently deposited in the Indian Museum, Calcutta (Bahuguna et al..,2023, Blanford, 1878). *Altiphylax stoliczkai* and *Altiphylax tokobajevi* have meager morphological difference. *Altiphylax stoliczkai* used in the present study for molecular study was from Ganglas, Leh Ladakh, India and the gene sequences of *Altiphylax tokobajevi* used in the present study were downloaded from NCBI. *Altiphylax tokobajevi* is known to be present in Eastern Uzbekistan.

2. MATERIALS AND METHODS

2.1 Study Area and Collection of Specimens

Road killed specimens (N=2) (Fig. 1) *Altiphylax stoliczkai* (Steindachner). Common name: frontier bow-fingered gecko, Baltistan gecko, or Karakorum gecko were collected from Ganglas, GPS GPS co-ordinates N 34.20247; E 77.61617 Alt: 3906 m asl, temp 17.1 C, humidity 19%). Ganglas is a locality present between Leh and South Pullu. The road killed specimens thus collected, registered (voucher number HARC/ZSI 257) and submitted to High Altitude Regional Center, Solan, Himachal Pradesh.

2.2 DNA Isolation

The tail samples were collected and cleaned with Milli Q water before digestion by incubating the dried tail sample for 24 hr in 1ml TE solution (Tris 10 mM and EDTA 1mM, PH 7.6) (Moraes-Barros and Morgant, 2007). After 24 hr of hydration the DNA was isolated from the tissue of tail using Himedia Forensic Kit.

2.3 PCR Amplification

COI gene sequences were amplified using a set of primer, LCO1490 (F) and (HCO2198 (R) (Vences et al., 2012). Polymerase chain reaction with initial denaturation of 94^0C for four minutes and each cycle of denaturation for 1 min at 94^0C, hybridization for 1 min at 55^0C ($42\text{-}45^0$C) and extension for 1 min at 72^0C was performed followed by final elongation for 10 min at 72^0C. The PCR cycle was repeated for 35 times for denaturation, hybridization and extension.

2.4 Gel Electrophoresis

The PCR products were visualized with 1.5% Agarose gel electrophoresis. Amplicons thus generated were sent for gene sequencing.

2.5 DNA Sequencing

The purified and PCR products were sent to Eurofin, Bangalore, India for sequencing. The amplicons (COI gene) were sequenced using Automated Sanger sequencing method on an automated ABI 3100 Genetic Analyser (device). COI gene sequences thus generated (Table 1) with forward and reverse primers, the consensus sequence were constructed and submitted to NCBI after conducting sequence alignment by BioEdit and by checking their similarity with species of genus *Altiphylax*. Rest of the gene sequences used in the present study were downloaded from NCBI (Table 1). PCR cocktail was prepared in PCR workstation (Bangalore GeNeiTM). Negative controls were used to check potential contamination. The sequences thus generated are submitted to NCBI after conducting sequence alignment by Bioedit and by checking their similarity with species of genus. The accession numbers obtained from NCBI are PP469571 and PQ002162.

2.6 Data Analysis

Sequences thus generated, were edited by using Chromas 1.6 (Technelysium Pty Ltd., South Brisbane Australia). To crosscheck, BLAST was used to compare DNA sequence data (12) All sequences were proof read and analyzed by using MEGA – XI (Kumar et al., 2018, Tamura et al., 2021) and were aligned by using ClustalW 9 (Thompson et al., 2003). MEGA- XI and DNAsp were used for finding the haplotypes, haplotype diversity, nucleotide diversity. Genetic data thus obtained were Eta: total number of mutations 134, Average number of nucleotide difference k 54.7, Nucleotide diversity Pi 0.08628, conservation sites, variable sites, parsimony informative sites. The COXI gene sequence of *Sphenodon punctatus* was used as an out-group for rooting the trees. Phylogenetic tree was generated by using MEGA XI based on Maximum Likelihood and Neighbour Joining for delimitation of the species. Both Maximum Likelihood and Neighbour Joining methods were used to know the consistency in topology of the phylogenetic tree.

Fig. 1. *Altiphylax stoliczkai* (Steindachner) in Ganglas, Leh, Ladakh

3. RESULTS

3.1 Genetic Data

The percentage genetic difference between the species *Altiphylax stoliczkai* Steindachner 1867 and *Altiphylax tokobajevi* (Jeremčenko & Szczerbak, 1984) for the COXI gene sequence is 20.166 %. The estimated genetic evolutionary distance between two species *Altiphylax stoliczkai* and *Altiphylax tokobajevi* was 7.5 to 7.9 (Table 4). Average nucleotide composition was noted to be 26.1 T(U), 30.9 C,23.0 A and 20.1 G.12 gene sequences thus analyzed have genetic data as given in Tables 2,3. There were 502 conserved sites, 134 variable sites, 134 Pi sites, 0 singleton out of 636 sites as analysed by MEGA XI. Number of haplotypes generated h: 4 with Haplotype diversity, Hd: 0.7121 as analysed by DNAsp (Tables 2,3).

Fig. 2. Phylogenetic tree of Altiphyax species by using Maximum Likelihood and Tamura Nei Model Accession numbers OR298054-60 (NCBI); OR298087 (NCBI) of COI gene sequences belong to *Altiphylax tokobajavi* displayed separate clade and PP469571,PQ002162, MZ293045 (NCBI) COX1 gene sequences formed a separate clade for species *Altiphylax stoliczka* Gene sequence of *Sphenodon punctatus* is used as an outgroup for the delimitation of the species

Table 1. Accession numbers of Genes sequences generated * and downloaded

Sample	Locality	Accession number COI
Altiphylax stoliczkai	Ganglas, Leh Ladakh, India	PP469571*
Altiphylax stoliczkai	Ganglas, Leh, Ladakh, India	PQ002162*
Altiphylax stoliczkai	NCBI	MZ293045
Altiphylax tokobajevi	NCBI	OR298053
Altiphylax tokobajevi	NCBI	OR298054
Altiphylax tokobajevi	NCBI	OR298055
Altiphylax tokobajevi	NCBI	OR298056
Altiphylax tokobajevi	NCBI	OR298057
Altiphylax tokobajevi	NCBI	OR298058
Altiphylax tokobajevi	NCBI	OR298059
Altiphylax tokobajevi	NCBI	OR298060
Altiphylax tokobajevi	NCBI	OR298087
Sphenodon punctatus #	NCBI	MN864229

**Novel data generated in this study; #Outgroup (Pyron et al., 2013, Skawiński and Bartosz 2017)*

Table 2. Species specific sites and Genetic data of the species examined by using MEGA XI and DNAsp

N	C	V sites	PI	S	Eta	k	Pi	Total
12	502	134	134	0	134	54.7	0.086	636

Number of gene sequences: 12, Conserved sites 502, Variable sites 134, Parsimony informative sites 134, Singleton 0, Eta: total number of mutations 134, Average number of nucleotide difference k 54.7, Nucleotide diversity Pi 0.08628

Table 3. Haplotypes detail as generated by DNAsp

S. No.	Haplotypes	Gene sequences creating haplotypes
1	Hap_1: 3	[PP469571.1 PQ002162 MZ293045.1]
2	Hap_2: 6	[OR298053.1 OR298054.1 OR298056.1 OR298058.1 OR298059.1 OR298087.1]
3	Hap_3: 1	[OR298055.1]
4	Hap_4: 2	[OR298057.1 OR298060.1]

Table 4. Estimates of Evolutionary Divergence between Sequences of species

	1	2	3	4	5	6	7	8	9	10	11	12	13
1PP459571 *Altiphylax stoliczkai*													
2PQ002162 *Altiphylax stoliczkai*	0.0												
3MZ293045 *Altiphylax stoliczkai*	0.0	0.0											
4OR298053 *Altiphylax stoliczkai*	7.9	7.9	7.9										
5OR298054 *Altiphylax stoliczkai*	7.9	7.9	7.9	0.0									
6OR298055 *Altiphylax stoliczkai*	7.9	7.2	7.9	0.0	0.0								
7OR298056 *Altiphylax stoliczkai*	7.9	7.9	7.9	0.0	0.0	0.0							
8OR298057 *Altiphylax stoliczkai*	7.9	7.9	7.9	0.0	0.0	0.0	0.0						
9OR298058 *Altiphylax stoliczkai*	7.9	7.9	7.9	0.0	0.0	0.0	0.0	0.0					
10 OR298059 *Altiphylax stoliczkai*	7.9	7.9	7.9	0.0	0.0	0.0	0.0	0.0	0.0				
11OR298060 *Altiphylax stoliczkai*	7.9	7.9	7.9	0.0	0.00	0.00	0.00	0.00	0.0	0.00			
12OR298087(2) *Altiphylax tokobajevi*	7.9	7.9	7.9	0.00	0.00	0.00	0.00	0.00	0.0	0.00	0.0		
13OR298087 *Altiphylax tokobajevi*	7.9	7.9	7.9	0.00	0.00	0.00	0.00	0.001	0.0	0.00	0.0`	0.0	

Altiphylax stoliczkai and Altiphylax tokobajevi.The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al 2004).

Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021)

Genetic distance: *The genetic distance between the two species noted to vary from 7.5 to 7.9*

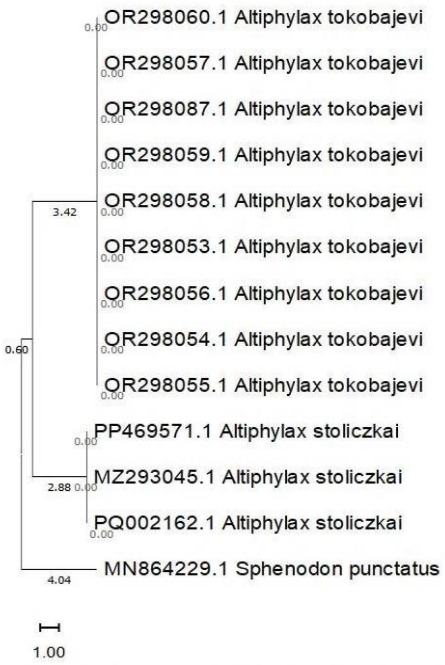

Fig. 3. Phylogenetic tree of Altiphylax species by using Neighbor-joining Method. Accession numbers OR298053-60 OR298087(NCBI), of COX1 gene sequences forms a separate dede for *Altiphylax tokobajevi* and PP469571, MZ293045, PQ002162 (NCBI) of COX1 formed separate dlade for *Altiphylax stoliczkai*. Gene sequence of *Sphenodon punctatus* was used as an outgroup for the delimitation of the species

3.2 Molecular Phylogenetic Analyses

Molecular phylogenetic analyses can provide insights into the evolutionary history of *Altiphylax stoliczkai* Steindachner 1867, including its relationships with other species within its genus or family. The results of the phylogenetic delimitation in the present study of the two species *Altiphylax stoliczkai* Steindachner 1867 and *Altiphylax tokobajevi* indicated the formation of the two clades in both Maximum likelihood and Neighbor joining methods (Figs. 2,3) with nesting of the two species into two separate clades.

4. DISCUSSION

A. stoliczkai is found in India (Kashmir, Karoo/Dras, Leh, Ladakh) and western China .The type locality was given by Steindachner is "*bei Karoo, nördlich von Dras, Kashmir* " (18). Palearctic naked-toed geckos belong to gekkonid geckos and their distribution range from North Africa to northern India and Western China. Their greatest diversity is noted to be in Iran and Pakistan. *Altiphylax* is a genus of small species of geckos, belonging to family Gekkonidae. They are endemic to Central Asia. But the relationships among the constituent genera remain incompletely resolved and the monophyly of key genera remains unverified. Two road killed specimens of *Altiphylax stoliczkai* Steindachner, 1867 were collected from Ganglas, Leh, Ladakh during survey of 15 days by the author.COX1 mitochondrial gene was used to differentiate the species of genus *Altiphylax* genetically. Five species of *Altiphylax* are known to be present globally and these are: *Altiphylax baturensis* (Khan and Baig, 1992), Batura Glacier gecko, *Altiphylax levitoni* (Golubev and Szczerbak,1979), Leviton's gecko;*Altiphylax mintoni* (Golubev & Szczerbak, 1981);*Altiphylax stoliczkai* Steindachner, 1867;*Altiphylax tokobajevi* (Jeremčenko & Szczerbak, 1984). Partial COXI gene sequences of *Altiphylax stoliczkai* Steindachner, 1867; *Altiphylax tokobajevi* (Jeremčenko & Szczerbak, 1984) were used to delimit the two species and also to genetically characterize the species. Two gene sequences of COXI of *Altiphylax stoliczkai* Steindachner 1867 were generated and gene sequences *Altiphylax tokobajevi* (Jeremčenko & Szczerbak, 1984) were downloaded from NCBI. Genetic data of the rest of the species are noted to be not available at NCBI. The preferred natural habitats of *A. stoliczkai* Steindachner 1867 are

desert grassland, and rocky areas, at altitudes of 2,300–3,700 m (7,500–12,100 ft) (Bahuguna et al., 2023, Khan et al., 2018). The distribution and habitat of the species was described by Bahuguna et al. in 2023. The species was reported from Gangalas, Leh, Ladakh during survey to Leh in 2019 (Bahuguna et al., 2023). The genus *Altiphylax* occurs in Central Asian region and *Altiphylax stoliczkai* Steindachner 1867 is found in desert, grassland and rocky areas of high-altitude regions. Both the species belong to Palearctic region. The tree depicts that *Altiphylax stoliczkai* Steindachner 1867 and *Altiphylax tokobajevi* (Jeremčenko & Szczerbak, 1984) formed the separate clades which means *Altiphylax stoliczkai* Steindachner 1867 and *Altiphylax tokobajevi* (Jeremčenko & Szczerbak, 1984) are distinct to each other genetically and the genetic difference was noted to be 20.166 %. and have distant common ancestor. There are two clades (Fig. 2) shown in the tree and the same result was noted with Neighbour Joining method (Fig 3). The phylogenetic analysis was done by using Maximum Likelihood method and Neighbor Joining Method form two clades with Tamura-Nei model with strong bootstrap with 500 replications (Figs. 2,3). One clade is having 9 sequences of *Altiphylax tokobajevi* and another clade is having two gene sequences of *Altiphylax stoliczkai*. The clade 1 (0.1397) of *Altiphylax tokobajevi* shows 100% bootstrap value with 500 replications (Figs. 2,3). The clade 2 (0.1058) of *Altiphylax stoliczkai* also shows 100% bootstrap value with 500 replications. Some molecular studies on high altitude lizards have been done to find out their phylogeny, to solve the taxonomic ambiguity and to explore the genetic basis of adaptation to high elevations in reptiles (Bauer et al., 2013, Krysko et al., 2007, Narayanan et al., 2022).

The present molecular study based on molecular marker mitochondrial Cytochrome c oxidase subunit I (COI) gene is useful in providing species specific molecular sites for identification of the species of genus *Altiphyalx*. The phylogenetic analysis also provides delimitation of the species of the genus and also indicates that *Altiphylax tokobajevi* is genetically distant to *Altiphylax stoliczkai* as they are present in different clade.

5. CONCLUSIONS

These finding have significant implications in conservation and taxonomy of the species. The study provides valuable insights into the molecular study of *Altiphylax stoliczkai* and *Altiphylax tokobajevi* for solving taxonomic problems of the species of the genus *Altiphylax* and also highlights the need for further research in this area especially to generate the genetic data for other species of *Altiphylax* to do various studies like adaptations to high altitude, solving taxonomic problems and phylogeny. The study indicates that COXI gene is adequate to delimit the species *Altiphylax stoliczkai* and *Altiphylax tokobajevi*. More genetic data needs to be generated for other species of *Altiphylax* with increase sample size for population study and for phylogeny and taxonomy.

DATA AVAILABILITY

Gene sequences data is available at NCBI (https://www.ncbi.nlm.nih.gov). Specimens were deposited at High Altitude Regional Center, Zoological Survey of India, Solan, Himachal Pradesh (https://zsi.gov.in>regional-centres, avtarkaur2000@gmail.com) under the voucher number HARC/ZSI 257.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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