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A first molecular study of *Alticola argentatus* (Severtzov, 1879) from Ladakh, India

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ABSTRACT: The present study is the first molecular characterization of *Alticola argentatus* from Ladakh, India. Eleven gene sequences of Cytochrome b were analysed from 7 species of the genus *Alticola*. Phylogenetic study and genetic distance were calculated by using MEGA XII. The phylogenetic study demonstrated that species *Alticola argentatus* from India and Russia nested in one subclades with genetic distance (0.00167) indicating that the species from India and Russia were evolved at the same time.

Keywords: *Alticola argentatus*, Cytochrome b, Ladakh, India.

INTRODUCTION

The silver mountain vole (*Alticola argentatus*) is a small rodent belonging to the family *Cricetidae* (Life of the planet Ladakh, 2020). They are distinguished by their silver-grey pelage, long vibrissae, rootless hypsodont molar and angular skull shape (Musser & Carleton 2005). There are more than 2000 different kinds of species of rodents, and approximately 30 families. They make up about 40% of all mammals' species (Smith, 2023; Nadachowski and Mead 1999). Rodents are mammals that have single pair of continuously growing incisors that never stop growing and are present in each of the upper and lower jaws (Carleton *et al.*, 2005). External and cranial measurements (in mm) are as follows: Total length of head and body, 87-128; length of tail, 36-64; length of hind foot, 16-21; length of ear, 13-18; condylobasal length, 23.3-29.5 adult body masses range from 20.8 to 54.6g (Davydov, 1988; Odinashoev, 1987; Rossolimo, 1989; Rossolimo and Pavlinov 1992).

This vole species is widely distributed across the Tien Shan and Pamir mountain ranges, encompassing regions of Kazakhstan, Kyrgyzstan, Tajikistan, Afghanistan, China, and Pakistan. Its northernmost range extends to the Saur Mountains, while the southernmost limit is recorded in the Gilgit region of northern Pakistan (Weiner and Gdrecki 1981).

The species is mainly found in the meadows and rocky screes of Ladakh, peculiarly on the slopes of the Tsokar plains at elevations of 3,500 - 4,700 meter (Life on the Planet Ladakh, 2020). The Himalayas, which are known as a biodiversity hotspot remain poorly studied in terms of rodent species assemblages. The North-Eastern region of India contains close to 65 per cent of the country fauna diversity in land mammals but the status of small mammals, particularly rodents from this biodiversity-rich region remains limited and insufficiently documented. The International Union for Conservation of Nature, (IUCN) Red List classifies the species as 'Least Concern' (LC) (Molur, 2016). The study of rodent genetics heavily depends on modern molecular tools such as DNA sequencing and genetic analysis. These methods helps to examine the genetic structure, evolutionary connections, behaviour, and disease vulnerability of rodents. Unlike earlier approaches that focused mainly on physical features and anatomy, molecular techniques have greatly advanced the classification and understanding of rodent species. Genetic research on small mammals also helps reveal how changes in the environment over time have shaped their movement patterns and genetic differences across different regions.

The present study deals with the molecular characterization and delimitation of the species so that the species could be differentiated at molecular level. This study is the first study of silver vole (*Alticola argentatus*) from India. To differentiate the species at molecular level Cytochrome b gene was used in the present study and the DNA source is road killed specimen of Silver vole from Ladakh.

MATERIALS AND METHODS

Sample Collection

Road killed specimen of Silver Mountain vole (*Alticola argentatus*) were collected during survey from Ladakh region of India (Fig. 1) by Dr. Anil Kumar, scientist of Zoological Survey of India. The specimens thus collected registered and submitted to Department of mammals, ZSI Dehradun. Tissue samples were collected randomly from the two mature healthy individuals of specimens *Alticola argentatus*. Additionally, Cytochrome b available sequences of species *Alticola* were also downloaded from GenBank (Table 1).

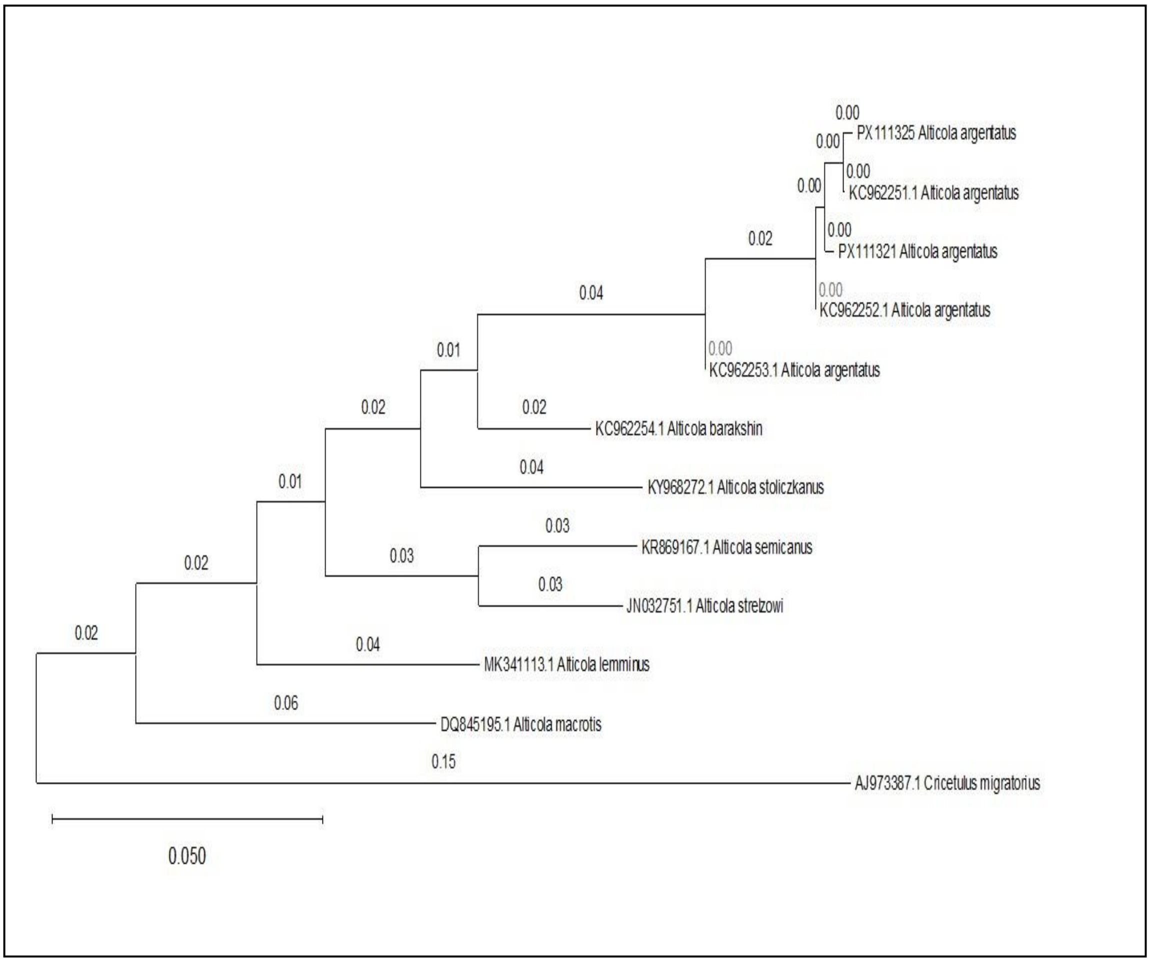

Fig. 1. Phylogenetic analysis using 11 gene sequences of Cytochrome b, with maximum likelihood algorithm.

Table 1: Source of sample collected, sample ID, registration No. their locality.

Species (common name)	**Sample**	**Locality**	**Accession number (cytochrome b)**
Alticola barakshin (Gobi Altai mountain vole)	*Alticola barakshin*	Mongolia	KC962254.1
Alticola stoliczkanus **(**Stoliczka's mountain vole)	*Alticola stoliczkanus*	Museum of Sichuan Academy of Forestry	KY968272.1
Alticola argentatus (Silver mountain vole)	*Alticola argentatus*	Ladakh, India	PX111321
Alticola argentatus (Silver mountain vole)	*Alticola argentatus*	Ladakh, India	PX111325
Alticola argentatus (Silver mountain vole)	*Alticola argentatus*	Zoological Institute Russian Academy of Sciences	KC962253.1
Alticola argentatus (Silver mountain vole)	*Alticola argentatus*	Zoological Institute Russian Academy of Sciences	KC962252.1
Alticola argentatus (Silver mountain vole)	*Alticola argentatus*	Zoological Institute Russian Academy of Sciences	KC962251.1
Alticola semicanus (Mongolian silver vole**)**	*Alticola semicanus*	Russia	KR869167.1
Alticola strelzowi (Flat-headed vole)	*Alticola strelzowi*	Kazakhstan	JN032751.1
Alticola lemminus (Lemming vole)	*Alticola lemminus*	Russia	MK341113.1
Alticola macrotis (Large-eared vole)	*Alticola macrotis*	Russia: Altai, Ulaganskii area	DQ845195.1

DNA isolation

Based on available tissue samples of the species, the tail samples were collected and cleaned with Milli Q water before digestion by incubating the dried tail sample for 24 hr 1ml TE solution (Tris 10mM and EDTA 1mM, PH 7.6). After 24 hr of hydration the DNA was isolated from the tissue of tail using Qiagen (Germany) MagAttract HMW DNA isolation kit were made for each individual (Bahuguna and Singh 2019).

PCR amplification

Cytochrome b gene sequences were amplified using a primer set of L14841 and H15149. (Kocher *et al.*, 1989).

Amplification of gene

Polymerase chain reaction consisted of initial denaturation of 94°C for 4 minutes and each cycle of denaturation for 1 minute at 72°C followed by final elongation for10 minutes at 72°C. The cycle was repeated for 35 times. The PCR products were sequenced using ABI's AmpliTaq FS dye terminator cycle sequencing chemistry on an automated ABI 3100 Genetic Analyser. All experiments were performed in a PCR workstation (Bangalore GeNeiTM). Negative controls were used in all DNA extraction and PCR amplification to control for potential contamination. Cytochrome b gene sequences thus generated are submitted to NCBI after conducting sequence alignment by Bioedit.

Data Analysis

CLUSTAL W sequence alignment tool was used for the alignment and comparison of genetic data by using BioEdit v 7.2.5.0 software (Hall 1990) with outgroup *Cricetulus migratorius* (for Cytochrome b). Proofread sequences were used and analyzed by using MEGA XII (Tamura *et al.*, 2011).

MEGA XII (Molecular Evolutionary Genetics Analysis) is widely used in bioinformatics for analyzing DNA and protein sequences. It helped us in finding conserved, variable, parsimony informative singleton sites as well as in construction of phylogeny.

The two methods used for phylogeny construction are (i) maximum likelihood and (ii) neighbor-joining method (Pevsner 2009).

RESULT

Cytochrome b gene was amplified from DNA extract of *Alticola* species i.e *A. argentatus, A. stoliczkanus, A. barakshin, A. semicanus, A. strelzowi, A. lemminus, A. macrotis.* After sequencing the apparent lengths of Cytochrome b (600bp) were obtained.

Genetic data

Species specific sites in Cytochrome b gene:

Table 2: Result obtained.

No. of Species examined	N	C	V	S	Pi	Hd
11	**600**	**456**	**144**	**73**	**71**	**11**

Mitochondrial gene of *Alticola argentatus* out of 600bp of Cytochrome b gene, there were 456 conserved sites and 144 variable sites and 73 singleton 71 parsimony informative sites 11 haplo-types and Haplotype diversity (Hd) (1.0000).

Genetic distance and base composition

The smallest genetic distance (0.00167) was observed between two samples of *Alticola argentatus* (PX111325 and KC962241.1). The largest genetic distance (0.13440) was found between *Alticola macrotis* (DQ845195.1) and *Alticola barakshin* (KC962254.1).

Nucleotide Composition of the gene sequences analyzed are as follows:

A= 27.9%, G=15.2%, T= 28.5%, and C= 28.4%

Phylogenetic analysis

Phylogenetic tree was constructed by MEGA XII software with 11 sequences, of *Alticola* species i.e. *A. argentatus, A. stoliczkanus, A. barakshin, A. semicanus, A. strelzowi, A. lemminus, A. macrotis* of 600 bp with *Cricetulus migratorius* (AJ973387.1) as outgroup.

DISCUSSION

The *Alticola* species are widely distributed across Central Asia, East Asia, and parts of South Asia, they are found in regions like the Tien-Shan, Pamir, Karakorum, and Hindu Kush mountains. *Alticola* is one of the least known groups of voles both in evolution and life history (Lebedev *et al.*, 2007). The genus *Alticola* comprises 12 recognized species (Pavlinov, 2003). Out of which *Alticola argentatus* which is geographically reported in the regions of Central Asia Tien Shan (China, Kazakhstan), Pamirs (Tajikistan/Kyrgyzstan), Hindu Kush (Afghanistan), northern Pakistan, Kashmir & Ladakh (India). The samples we worked upon are of species *Alticola argentatus* commonly known as silver mountain vole largely found in the Ladakh region of India. The road killed species were collected from the field during Ladakh survey (Fig. 2).

Cytochrome b genes of several species of *Alticola* were downloaded on NCBI across different localities and the gene sequences of *Alticola argentatus* were compared with other species in the genus. As *Alticola barakshin* found in Mongolia, northern China and Southern Siberia (Russia). *Alticola stoliczkanus across* Western China (Tibet, Xinjiang), Pakistan, Nepal. *Alticola lemminus* found in Central and southern Siberia (Russia). *Alticola strelzowi* has been reported in Mongolia, northern China, Kazakhstan, and southern

Siberia (Russia), and *Alticola macrotis* is found in Mongolia, as well as southern Siberia (Russia).

Genetic distance and Genetic data. The smallest genetic distance (0.00167) was observed between two samples of *Alticola argentatus* (PX111325 and KC962241.1), indicating they belong to the same species and may originate from the same population. In contrast, the largest genetic distance (0.13440) was observed between *Alticola macrotis* (DQ845195.1) and *Alticola barakshin* (KC962254.1) suggesting they are very distantly related.

Fig. 2. Silver mountain vole, ladakh (jammu & kashmir).

Phylogenetic delimitation of the species. The maximum-likelihood (ML) phylogenetic tree based on 11 mitochondrial cytochrome *b* gene sequences provides clear insights into the genetic relationships and lineage diversification within the genus *Alticola*. The tree is rooted with *Cricetulus migratorius* (AJ973387.1), which serves as an outgroup.

The phylogenetic tree shows that *Alticola macrotis* (DQ845195.1), from Russia, diverges as a single lineage at the base of the tree, forming the earliest branch within the genus. This long branch length (0.15) suggests that *A. macrotis* is genetically distinct from other *Alticola* species, possibly representing an ancestral or highly divergent lineage.

Both Indian sequences from Ladakh (*Alticola argentatus*) PX111321 and PX111325 clustered with negligible genetic distances (0.00 to 0.01). The internal bootstrap values further support this strong genetic relatedness — 97% between PX111325.1 and KC962252.1, 100% between PX111321 and KC962251.1, and 87% at the node connecting these sequences. This clearly indicates high genetic similarity and minimal divergence between *A. argentatus* individuals from India (Ladakh) and Russia, suggesting a shared evolutionary history and likely recent divergence. It is possible that these populations were once part of a continuous distribution and have only recently become isolated. Also, the gene sequence KC962253.1, *Alticola argentatus*, from Russia show closeness with the other *Alticola argentatus* sequences (Fig.1).

Alticola barakshin (KC962254.1) was noted to be genetically close to *Alticola argentatus* as revealed by the phylogenetic tree and genetic distance (Fig. 1). Another distinct subclade includes *Alticola semicanus* (KR869167.1) and *Alticola strelzowi* (JN032751.1). These two species cluster together with strong bootstrap support and branch lengths of 0.03, indicating moderate genetic divergence but a shared recent common ancestor. Their close relationship, perhaps influenced by geographic separation or ecological divergence in Central Asia.

CONCLUSIONS

Eleven gene sequences of Cytochrome b thus analysed from 7 species of the genus *Alticola* by using phylogenetic delimitation and genetic distance by using MEGA XII demonstrated that species *Alticola argentatus* from India and Russia nested in one subclade with genetic distance of 0.00167. Thus, study inferred the co evolution of the species from India and Russia. Other species of *Alticola* however also delimitated by using Cytochrome b gene with separate lineages.

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