Final Project Update

(August 2023- December 2024)

Progress during period:

During this period multiple awareness programmes has been conducted to spread the information about the ecological importance of Bengal Monitor Lizard, the taxonomic keys to identify between the extant species of India and cleared the myths and misconceptions about the usage of body parts of Bengal Monitors.

Following are details of each programs:

1. Master's student visits in Wildlife Institute of India from Panjab University (March, 2024)


2. Master's student visits in Wildlife Institute of India from T.N College (May, 2024)


3. Infographic poster pasting at local tea shops and market areas by Mr. Amit Badola and Mr. Devendra Rawat in Koti Kanasar, Chakarata (June, 2024)

4. Master's student visits in Wildlife Institute of India from PGP College of Arts & Science, Tamil Nadu (June, 2024)

5. Had an opportunity to present about Bengal Monitor lizard at 10th World Congress of Herpetology, Borneo in oral presentation section entitled "When biogeography meets forensics: Case study with Bengal monitor lizard (*Varanus bengalensis*)" (August, 2024)

6. A whole day workshop in Sundarvan Mini Zoo in Ahmedabad, Gujrat for visitors ranging from Children to Adults regarding the Ecology and behaviour of Bengal Monitor Lizard. Sundarvan Zoo hoses 4 Bengal Monitor Lizard and one of the famous places in Ahmedabad. The workshop is assisted by the Park Manager Mr. Deven Mehta. (September, 2024)

7. Awareness function among school children at Gwasa Pul Village in Chakrata region of Dehradun District (October, 2024)

Throughout the project, information about Bengal monitor lizards was gathered from various individuals and groups. One of the primary research queries focused on whether local communities could differentiate between juvenile and adult Bengal monitors. Unfortunately, most respondents identified juveniles and adults as entirely different species. Additionally, numerous anecdotes and narratives about monitor lizards were documented during the study. These stories provide valuable cultural and behavioural insights and are being compiled for publication as a short communication (work in progress).

A group of wildlife enthusiasts has been trained to record GPS locations and capture photographs of monitor lizard encounters, with the data being uploaded to platforms like iNaturalist and GBIF. To further this citizen science initiative, a dedicated

WhatsApp group has been created to facilitate real-time communication and data sharing. WhatsApp is widely accessible, requiring minimal training for users, as most participants already own smartphones and are familiar with the platform. This group serves as an effective tool for promoting conservation efforts by enabling the swift exchange of information and fostering collaboration among participants, ultimately strengthening evidence-based conservation initiatives.

As the part of awareness, t-shirts were printed as a promotional material containing the Rufford logo and a tag line. These t-shirts have been distributed among ground staff of forests, members working for Monitor lizards in different NGOs, among wildlife enthusiast and Forest officials as token of appreciation.

Molecular Output (Private report)

The following section of the report consist phylogenetic outcomes of the part of this project and my PhD objective. **The following outcomes will be communicated as a paper in selected peer-reviewed journal**. Hence it is a humble request to The Rufford Foundation **for not uploading the following figures and result provided.**

DNA Extraction:

Tissue collected from individuals was stored in 70% ethanol in field. The samples were chopped finely for lysis procedure followed by extraction through Qiagen Blood and tissue kit. The extracted DNA was then quantified through gel electrophoresis.

PCR amplification:

Mitochondrial gene Cytochrome b (Cyt *b*), NADH dehydrogenase (ND5) and Cytochrome c oxidase subunit I (CO*I*) was successfully amplified on few samples. Cyt *b* was also amplified for the confiscated hemipenses for the matching purposes. Microsatellite loci (n=6) were also genotyped for same samples.

Sequencing & Genotyping:

The amplicons were then used for sequencing and genotyping through ABI 3500XL.

Data Analysis:

The mitochondrial data was analyses using various software. DnaSP ver 6 was used to compute the number of haplotypes among the generated dataset. The data matrix was executed in MEGA X to determine an appropriate model for maximum likelihood analysis. A Monte Carlo Markov Chain (MCMC) based Bayesian consensus tree was constructed using BEAST 1.7.

Results:

Interestingly, in *V. bengalensis* I observed two genetic signatures which clearly differentiated the species into two different lineages: Lineage I and Lineage II. The details of SNP variation in both lineages were shown in Table 1. In total, 22 polymorphic sites which consisting 26 parsimoniously informative sites and 7 singleton sites were

observed in COI (977bp) gene (Figure 1). In ND5 (668bp) gene, there were 34 polymorphic sites consisting 40 parsimoniously informative sites and 8 singleton sites (Figure 2). However, in Cyt b (814bp) gene, there were 23 polymorphic sites consisting 32 Parsimoniously Informative sites and 8 Singleton sites (Figure 3). Frequencies of nucleotide composition of different monitor lizard are represented in (Table 2).

Table 1: Polymorphic site within *V. bengalensis* from different mtDNA genes

Species	Gene	SNP position
V. bengalensis_Lineage I	COI	5240, 5285, 5330, 5405, 5474, 5558, 5594, 5861, 5900, 6059, 6089
V. bengalensis_ Lineage II		5411, 5738, 5891, 5909, 5945, 5954, 5960, 6002, 6023, 6062, 6110
V. bengalensis_Lineage I	ND5	13323, 13363, 13385, 13508, 13559, 13594, 13595, 13598, 13631, 13674, 13727, 13787, 13826, 13878, 13910, 13914, 13931
V. bengalensis_ Lineage II		13469, 13520, 13538, 13559, 13586, 13652, 13671, 13709, 13730, 13756, 13766, 13799, 13911, 13914,13917, 13922, 13928
V. bengalensis_Lineage I	Cyt b	14074, 14089, 14104, 14158, 14185, 14242, 14248, 14338, 14482, 14521, 14636, 14699, 14722
V. bengalensis_ Lineage II		14248, 14274, 14282, 14383, 14476, 14551, 14590, 14614, 14681, 14737

Table 2 Nucleotide composition of different monitor lizard species using mtDNA

Common Name	Scientific Name	No. of Sample	Gene	T(U)	C	A	G
Bengal monitor lizard-Lineage I	*V. bengalensis*	25	COI	27.9	30.3	30.1	11.6
			ND5	26.0	33.0	33.2	7.8
			Cyt b	27.9	30.3	30.1	11.6
Bengal monitor lizard-Lineage II	*V. bengalensis*	48	COI	27.0	31.3	30.2	11.6
			ND5	25.8	33.3	33.1	7.7
			Cyt b	27.0	31.3	30.2	11.6
Yellow monitor lizard	*V. flavescens*	9	COI	27.4	31.1	29.2	12.3
			ND5	27.5	31.8	32.1	8.6
			Cyt b	27.4	31.1	29.2	12.3
Desert monitor lizard	*V. koniecznyi*	2	COI	26.0	32.5	29.0	12.5
			ND5	22.6	37.5	31.2	8.6
			Cyt b	26.0	32.5	29.0	12.5

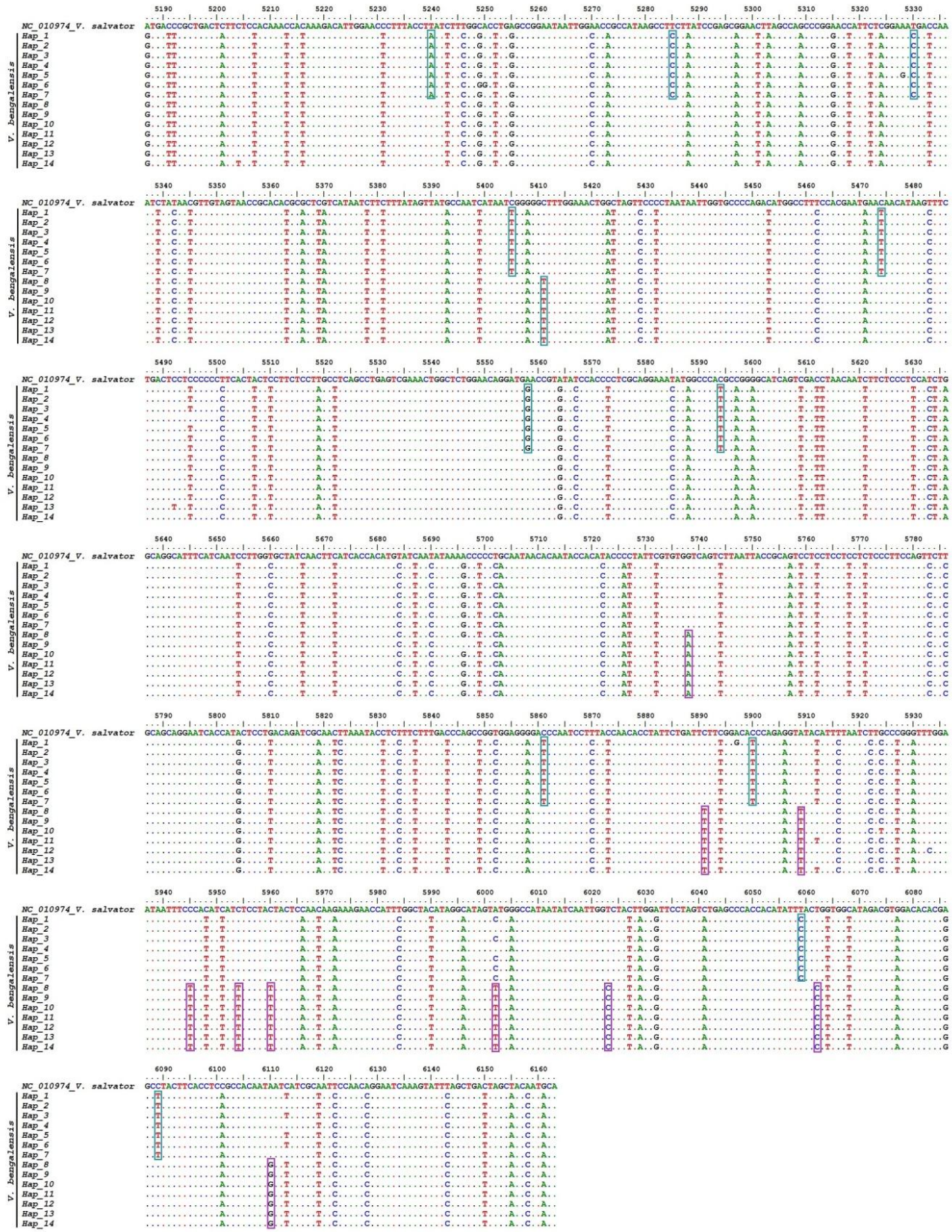

Figure 1: Single Nucleotide Polymorphism (SNPs) positions within *V. bengalensis* in COI gene, nucleotide positions calculated based on the complete mitochondrial genome of *V. salvator* (NC010974)

Figure 2: Single Nucleotide Polymorphism (SNPs) within *V. bengalensis* in ND5 gene, nucleotide positions calculated based on the complete mitochondrial genome of *V. salvator* (NC010974)

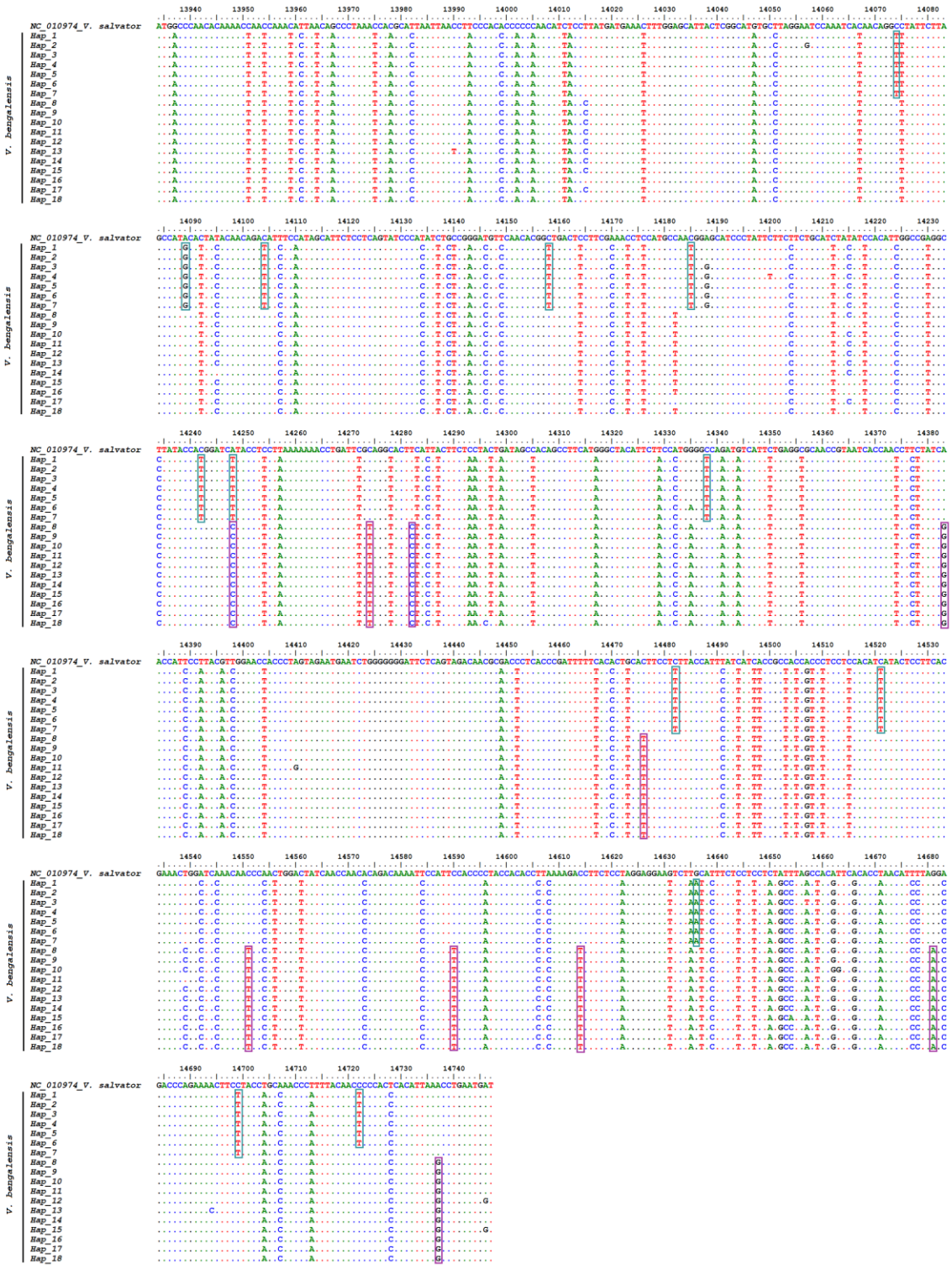

Figure 3: Single Nucleotide Polymorphism (SNPs) within *V. bengalensis* in Cyt *b* gene, nucleotide positions calculated based on the complete mitochondrial genome of *V. salvator* (NC010974)

Phylogenetic trees derived from COI, ND5 and Cyt *b* genes indicated well-supported clades with posterior probability values (>0.6). The resulting tree of ND5 and Cyt *b* genes showed the presence of two well-supported lineages within *V. bengalensis*: Lineage-I and Lineage-II. Conversely, the resultant tree of COI gene showed the

presence of three well-supported clades within *V. bengalensis*: Lineage-I and Lineage-II show congruence with the ND5 and Cyt b phylotrees, whereas Lineage-III consisted of the sequences from South India taken from NCBI database. Due to the unavailability of ND5 and Cyt *b* sequences from South India, we could not further assess their evolutionary relationships (Figure 4). {More than 60% of sampling was done before the award date of The Rufford grant}

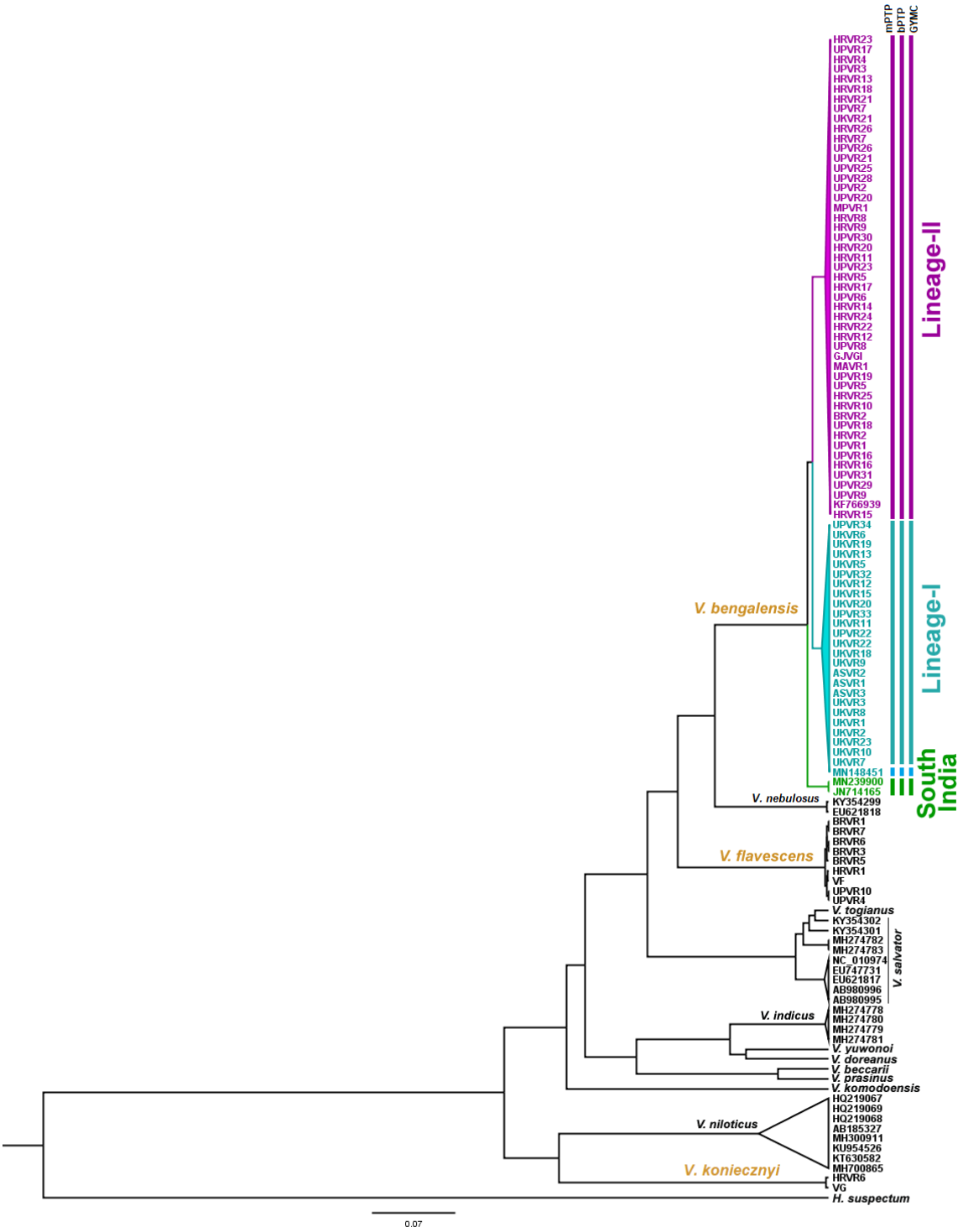

Figure 4: Bayesian inference (BI) of phylogenetic tree for genus *Varanus* based on COI gene. Tree also represents the result of three different molecular species delimitation methods.

The established baseline genetic database has been instrumental in matching confiscated hemipenes received at the Wildlife Institute of India. Due to the degraded condition of the samples, mitochondrial Cyt *b* gene analysis was primarily employed, supplemented by genotyping for microsatellite loci. The results indicate that the confiscated samples show genetic affinity to either of the two lineages of the Bengal monitor lizard (Figure 5).

This identification provides crucial insights for enforcement agencies, highlighting major conduits for the illegal trade. Notably, a significant number of confiscated samples were traced to Lineage II of the Bengal monitor lizard, which is predominantly found in plains-like topography rather than the Himalayan foothills. This finding underscores the role of smooth terrain and an extensive transportation network in facilitating poaching activities and illegal trade.

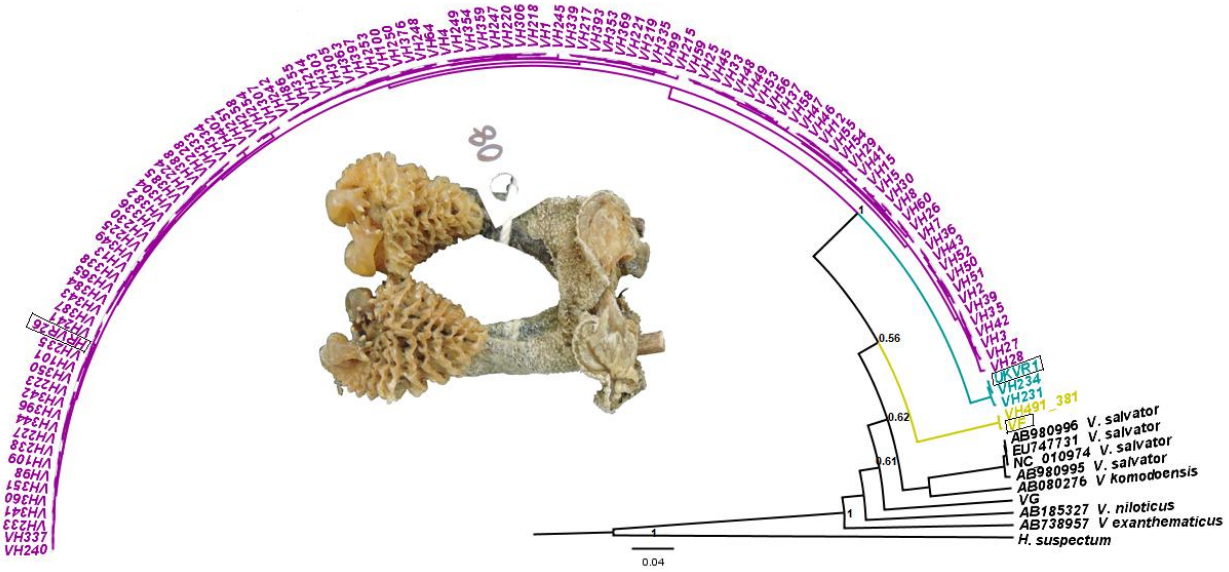

Figure 5: Bayesian inference (BI) of phylogenetic tree for confiscated samples based on Cyt *b* gene. Reference sample of different monitor lizard is in black box

A TCS Network was also constructed with the dataset of Cyt *b* gene that comprises references database and dataset derived from the confiscated samples. Interestingly, the spatial distribution of haplotypes from confiscated samples closely aligned with the phylogenetic relationships observed among the distinct clades of monitor lizards. The TCS network showed two major and one minor cluster corresponding to the phylogenetic clades: *V. bengalensis* Lineage-I, *V. bengalensis* Lineage-II and *V.*

flavescens. The *V. bengalensis* Lineage-I has a significant divergence from *V. bengalensis* Lineage-II by ten mutational steps (Figure 6). On the other hand, *V. flavescens* group has noteworthy divergence from *V. bengalensis* group by twenty-two mutational steps. Additionally, there were moderately fewer substitutions separating sub-groups within the major groups, indicating genetic coherence within groups of *V. bengalensis* Lineage-I, *V. bengalensis* Lineage-II and *V. flavescens*.

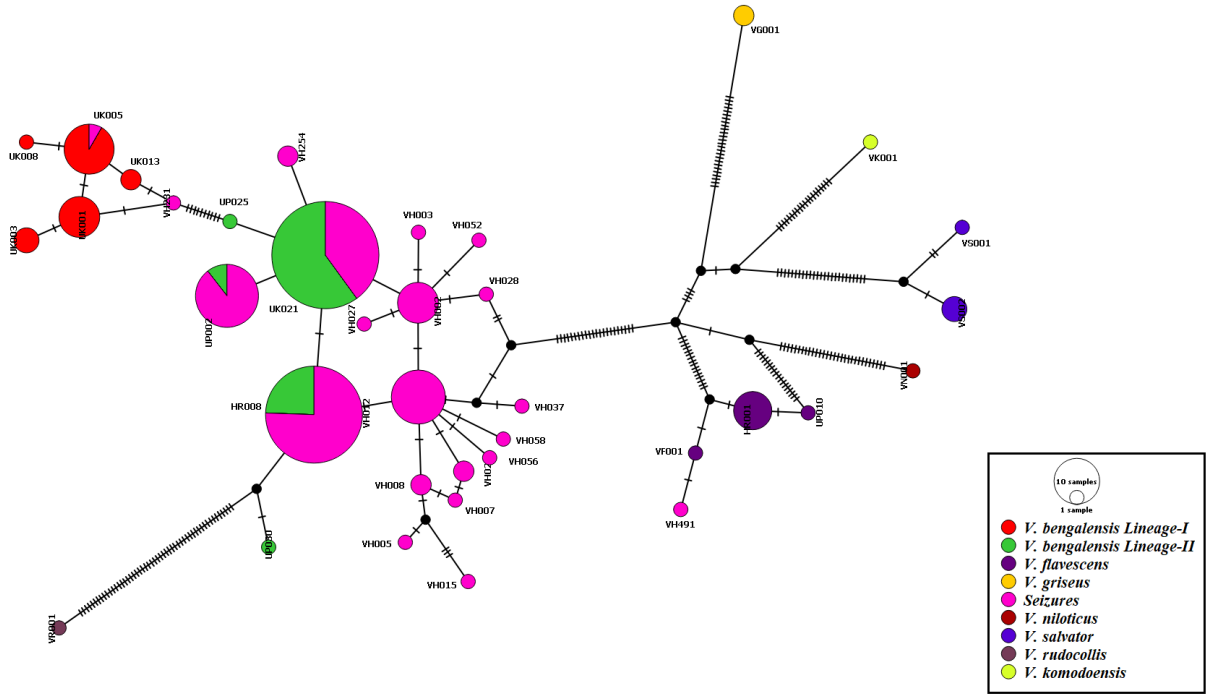

Figure 6: Confiscated and reference haplotype network based on TCS method for the 302 bp mtDNA fragment of Cyt *b*. Circles denote individual haplotypes, with their sizes indicating the relative abundance of each haplotype

The microsatellite (STR) database of 84 individual monitor lizard were generated which were geo-tagged reference samples comprising *V. bengalensis* (n=73), *V. flavescens* (n=9) and *V. koniecznyi* (n=2) from six loci. These same six loci were used to generate STRs for confiscated samples (n=114). Further analysis was done by combining the dataset of reference sample and confiscated sample. The multivariate DAPC corroborated the Bayesian clustering analysis that differentiated the populations into four genetic clusters and clustering of seizures with *V. bengalensis* (Lineage-I: Himalayan foothills), *V. bengalensis* (Lineage-II: Remainder of Mainland) and *V. flavescens* (Figure 7). A parallel pattern between genetic cluster assignment and phylogenetic clades was observed.

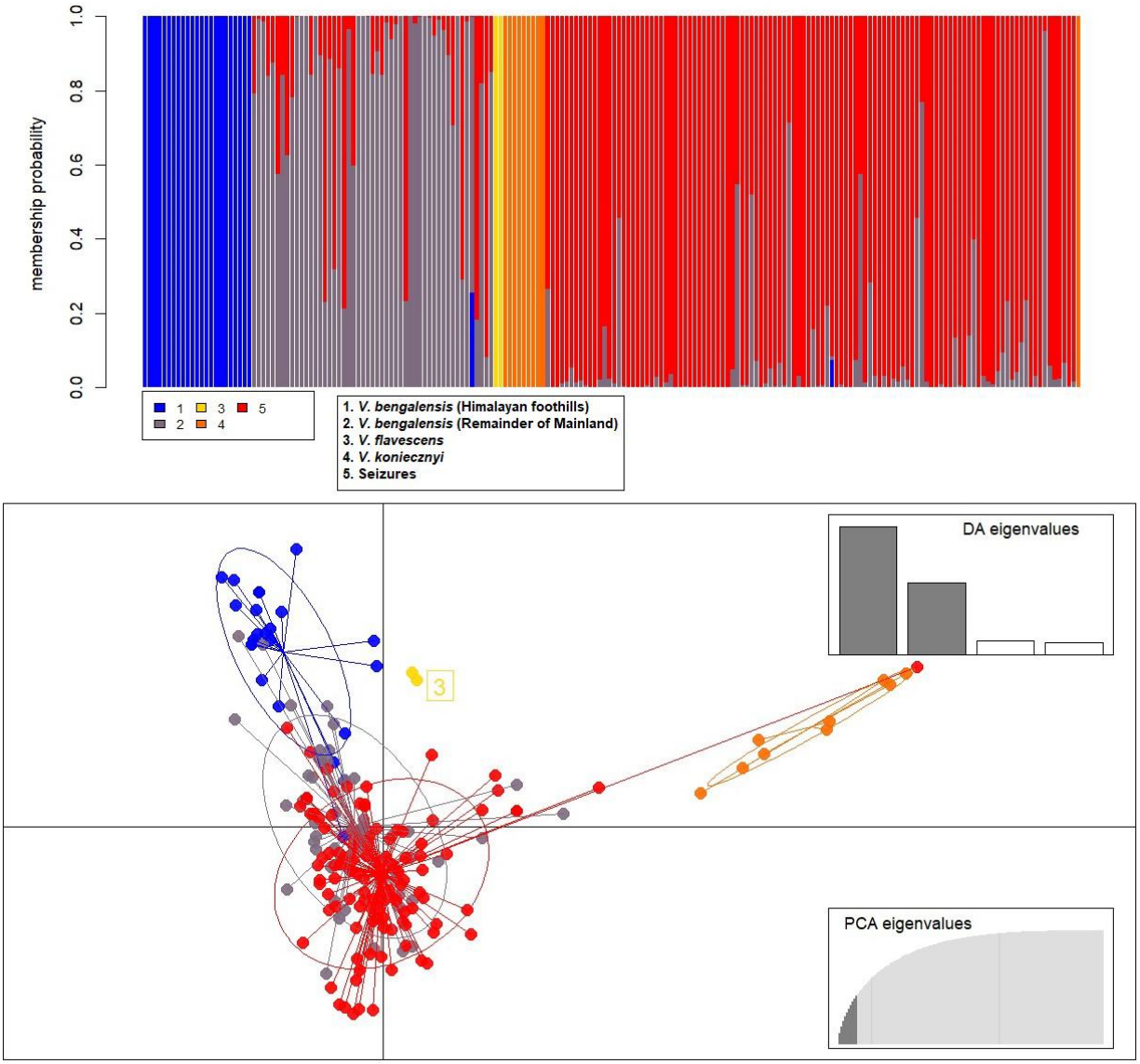

Figure 7: The bar plot results show genetic clustering implemented in Discriminant Analysis of Principal Components (DAPC) (a). Each column along the X-axis represents one *Varanus* individual. The Y-axis represents the assignment of the membership probability of each individual. Scatter plots of DAPC using a hierarchical islands model and shown by different colors and inertia ellipses (b). The DA and PCA eigenvalues of the analyses are displayed in the inset

The baseline genetic database of reference samples has established as distinct signature for monitor lizard species from Terai Arc landscape and associated landscape (Figure 8). The methodology adopted proposes a framework for the recognition of monitor lizard species from the confiscated samples which would facilitate the implementation of standardized protocol for molecular based species identification tool that help wildlife forensics to track the origin of species and poaching hotspots.

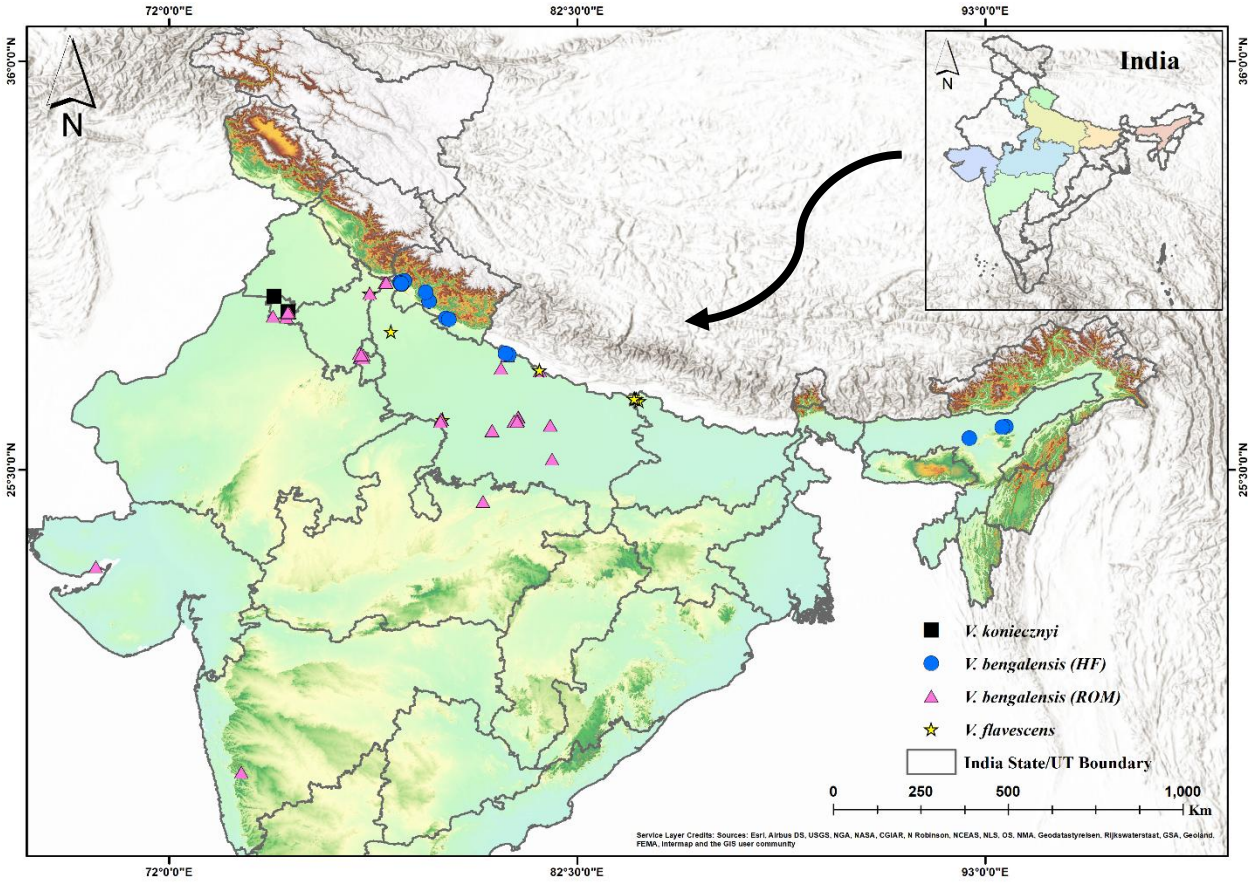

Figure 8: Reference Geo-tagged sampling points with the identity of specific species cluster