PHYLOGENETIC STATUS OF SAMBAR *Rusa unicolor* IN WESTERN GHATS

Phylogenetic Status of Sambar (*Rusa unicolor*) in Western Ghats

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Principal Investigator

S. K. Gupta
Scientist-D

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

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Project Team
S.K. Gupta, Principal Investigator
Nipun Mohan, Technical Assistant
Ajit Kumar, Volunteer

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(Final Report)

1.	**Project Title:**	Phylogenetic Status of Sambar (*Rusa unicolor*) in Western Ghats
2.	**Duration of the Project**	18 months
3.	**Name and Designation of Investigator**	S.K. Gupta, Scientist-D, WII skg@wii.gov.in
4.	**Name of the research fellow**	Nipun Mohan (Technical Assistant)
5.	**Final budget**	Rs 11,40,000.00
6.	**Name of the funding agency**	Wildlife Institute of India, Dehradun
7.	**Date of submission of final report**	4 August, 2014

8. List of degrees awarded

(i) Ph.D. thesis-

Sandeep Kumar Gupta, Wildlife Institute of India submitted thesis to University of Saurashtra, Gujarat.

(ii) M.Sc. dissertation-

Ms. Neha Saini awarded M.Sc. in Biotechnology from HNB Garhwal University.

(iii) B.Tech dissertation-

a. Ms. Komal Rani awarded B.Tech in Biotechnology from Jayoti Vidyapeeth Women's University, Jaipur.

b. Ms. Neha Singh, awarded B.Tech in Biotechnology from Amity University, Noida, Uttar Pradesh.

9. List of publications

a. Gupta SK, Kumar A and Hussain SA (2014) Novel primers for sequencing of the complete mitochondrial cytochrome b gene of ungulates using non-invasive and degraded biological samples. *Conservation Genetics Resources* **6**: 499-501.

b. Gupta SK, Kumar A and Hussain SA (2013) Extraction of PCR-amplifiable DNA from a variety of biological samples with uniform success rate. *Conservation Genetics Resources* **5**: 215-217.

EXECUTIVE SUMMARY

Phylogenetics is the study of genetic relationships between species using molecular sequencing data. Phylogeography is an advanced method being used in resolving population substructure at geographical scales and fine taxonomic levels for better conservation planning. It depends upon the assessment of genetic variation across large landscape for species having wide distribution range. Sambar (*Rusa unicolor*) is one of the most-suitable model ungulate for such study. It is a resident of most of the biogeographic zones of India. It was established by genetic analysis that the Javan rusa (*R. timorensis*) of Indonesia is probably the closest living relative of the sambar. It is also essential to know the phylogenetic variations (if any) among sambar population in India for identification of population(s) for proper conservation management. Therefore, the following objectives were set forth: (a) Molecular insight into taxonomy of sambar, by using potential mitochondrial DNA (mtDNA) markers (b) To examine the genetic variation of sambar population in Western Ghats by using potential mtDNA and STR markers. This study was focused on the examination of intra and inter-species genetic variation among Indian sambar (*R. u. unicolor*) populations using mitochondrial DNA (mtDNA) and nuclear DNA markers. Sequence variation in a partial fragment of mtDNA control region was examined from the biological samples collected from Western Ghats, Deccan Peninsula, Semi-arid and Gangetic plains. After alignment, the sequences generated from the 23 samples of Western Ghats exhibited a 40 bp insertion after nucleotide (nt) position 233. This unique insertion in a majority of the Western Ghats samples indicated significant genetic variation in this population. This insertion was not observed in the samples from Deccan Peninsula, Semi-arid, Gangetic plains populations and few individuals from the Western Ghats populations. Therefore, insertion-deletion (INDEL) in the control region after nt position 233 appears to be a potential marker for genetic screening of these populations. Overall, the mtDNA control region demonstrates high variability among sambar populations, and 26 distinct haplotypes were identified.

The DNA sequences were also generated for mtDNA cytochrome *b* (cyt *b*) gene from the sambar populations across different biogeographic zones of India. Phylogenetic analysis based on the cyt *b* gene sequence exhibited clear-cut genetic variation among sambar populations and segregated them into two genetically distinct clusters. One of the clusters is largely distributed from north to south India in Gangetic Plain, Semi-Arid, Deccan Peninsula and few of the Western Ghats individuals, and the other cluster was restricted mainly to Western Ghats. Based on similarity among 77 in-house and 11 GenBank sequences of *R. unicolor* and two sequences of Javan sambar (*R. timorensis*), 21 haplotype were identified using DnaSP program and named as S1-21. The haplotype S1-19 were of recognized *R. unicolor*, and S20-21 were of *R. timorensis*. While examining the sequence variation in cyt b of all sambar samples, an interesting disparity was observed in select sambar population in the Western Ghats. Three haplotypes labeled as S1-3 were exclusive for Western Ghats. Rest 15 haplotypes of sambar were from remaining part of the sampling locations of India and from overseas. The Neighbor-Joining (NJ) trees demonstrated four distinct clusters within the 21 haplotypes of sambar (Genus Rusa) and labelled as G1-4. Major cluster of sambar was G1, which appeared to be distributed from north to south of its range including the populations from Gangetic Plain, Semi-Arid, Deccan Peninsula and few individuals from the Western Ghats. Second cluster, G2 consists of two sequences each of Javan sambar and sambar from Thailand. Third cluster, G3 shows interesting similarity of eastern sambar of India with that of the Malayan subspecies. The last cluster, G4 was exclusive of Western Ghats and not observed from the samples collected from other part of India. These analyses indicated that G4 appeared to be a unique cluster. Evaluation of sequence variation demonstrates an unambiguous 4% variation of G4 with that of G1, G2 and G3. The extent (4%) of genetic variation in evolutionary conserve gene (cyt b) within a species invites further attention to look into the phylogenetic status of these populations. This variation could be an indication that G4 is an evolutionary significant unit.

Based on genotyping data obtained from 23 microsatellite loci, genetic variability was estimated to measure the level of heterozygosity. Overall, the mean number of alleles per locus was 6.65, and mean proportion of individuals typed was 0.56. It showed that the markers used for this study were appropriate for assessment of genetic fitness in sambar populations. The mean polymorphic information content (PIC) value was 0.653 indicating that tested loci were highly polymorphic for

sambar species. The mean expected and observed heterozygosity was 0.720 and 0.533, respectively. The observed heterozygosity was slightly lower than the expected value.

Couples of novel and pioneer finding have also been recorded during this research work. Couple of research methodologies was set forth for future study on related ungulate species. A variety of biological samples was used in this study for evaluation of genetic variations. The first and important step was extraction of good quality DNA from these samples. Therefore, three protocols were compared for extraction of PCR amplifiable DNA from bones, antlers and faeces samples of sambar. These protocols were Phenol-Chloroform (PC), column based Qiagen kit, and Guanidine hydrochloride (Gu-HCl) based in-house method. The effectiveness of the protocols was compared for higher PCR amplification success rate for the extracted DNA. These DNA extraction protocols yielded different PCR outcomes. PCR amplification in bone samples was negligible in PC method, low in Qiagen kit and high with Gu-HCl method. Poor amplification was observed for DNA extracted from bone, antler, and feces using PC method. In antlers, PCR amplifications were detected in all the three methods. For fecal samples, Qiagen Stool kit and Gu-HCl method show comparable PCR amplification. The PC method was found inconsistent with the fecal samples. Uniform PCR success rate and sequencing evidenced that Gu-HCl method is fast, low-cost, and less hazardous. It highlighted that the silica-based indigenous DNA extraction protocol using Gu-HCl chaotropic salts yields better quality DNA with higher PCR amplification success rate.

For successful amplification of the complete mitochondrial cytochrome *b* (cyt *b*) gene of ungulate species, a set of novel primers was described from this study. DNA extracted from non-invasively obtained and decomposed samples is found to be degraded and inappropriate for amplification of a complete gene (more than 1 kb) in single PCR amplification. Hence, a series of six ungulate-specific conserved primers for the cyt *b* gene was developed for sequencing of complete gene. These primers, in various combinations, amplify smaller fragments for these genes. Complete consensus sequences derived from these smaller fragments provides complete gene sequence. It was also used in multiplex PCR reaction to obtain the maximum possible size of the PCR amplification product.

In this study, one evolutionary significant unit (ESU) of sambar was identified from Western Ghats. However, further study needs to be conducted for their detailed ecological feature and barrier, which support their restricted distribution in this region. This ESU may require dedicated attention for proper management. It also provided scope to evaluate the variations at genetic or morphological level in other sympatric or widely distributed species.