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Original Article

Cytochrome B Variation among the Subspecies of *Antigone antigone*

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Abstract

Antigone antigone (Linnaeus, 1758), commonly known as Sarus Crane is a widely distributed water bird that is found in India, Australia, and Southeastern parts of Asia. It is divided into three extant subspecies, but its number has been on a decline in recent years. In this study, a non-invasive approach was applied for obtaining the genomic DNA from the molted feathers extracted from Sarsai Nawar Wetland, Etawah, Uttar Pradesh. The genetic diversity was evaluated from the 429 bp long mitochondrial DNA cytochrome b as a genetic marker was sequenced and evaluated for its genetic diversity. Hap2, encompassing sequences from India and Australia, emerged as the most prevalent haplotype in the sample. The degree of genetic difference between the subspecies exhibited a F_{ST} value of 0.391. The entire sample of Sarus Cranes had a low number of cyt b haplotypes, but the subspecies still had a high degree of genetic diversity. Negative, non-significant values for Tajima's D (-1.660) indicate neutral evolution of populations of the three subspecies.

Keywords: *Biodiversity, Cytochrome B Diversity, Genetic Conservation, Sarus Crane, Sarsai Nawar Wetland*

Introduction

The Indian Sarus Crane is the tallest flying member of the family Gruidae which is categorized into balearicinae (Crowned Cranes) and gruinae (Typical Cranes) based on the presence of a coiled trachea and unison call patterns (Archibald, Gopi Sundar & Barzen, 2003; Gopi Sundar, 2019). The Sarus Cranes (*Antigone antigone*) inhabit two continents, Australia and south eastern regions of Asia. Four subspecies, the Indian Sarus (*A. a. antigone*), the Eastern Sarus (*A. a. sharpii*) and the Australian Sarus (*A. a. gillae*) were initially differentiated on the grounds of morphology, plumage and geographical isolation. However, the Philippine Sarus, *G. a. luzonica* is now considered extinct (Archibald, Gopi Sundar & Barzen; 2003; Nevard *et al.,* 2020). The Sarus Crane has faced considerable extirpation of its range in the past consequently, the Indian subspecies is listed in the Vulnerable category of the IUCN red list (Gopi Sundar, Kaur., & Choudhury, 2000, CITES appendix 2016). This loss is a result of many factors including loss and degradation of wetlands, ingestion of pesticides, shifting to modern agricultural practices, hunting of adults, eggs and chicks for trade, food, and medicinal purposes. In the Indian subcontinent, the Sarus population is estimated to be 15,000-20,000 individuals (Gopi Sundar 2019), 800-1,000 in Cambodia, Laos and Vietnam, 500-800 in Myanmar, and about 10,000 breeding adults in Australia (Garnett & Baker 2022), building the population size of around 19,000-21,800 individuals. Population declines are often accompanied by the loss of genetic diversity (Hedrick 2011) and according to a recent study low genetic diversity in birds poses an increased extinction risk (Canteri *et al.,* 2021) (Hvilsom *et al.,* 2022).

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Despite reports of population declines of Sarus Cranes and the importance of genetic studies to biological conservation, only a couple of studies have attempted to report on the genetic variation of *A. antigone* across their entire distribution. A limited number of studies were conducted for some areas occupied by *A. antigone* (Wood & Krajeswki 1996, Jones, Barzen & Ashley 2005, Nevard *et al.,* 2020). In light of their recent global population declines (Gopi Sundar 2019), we need genetic conservation for which cytochrome b gene serves as an indispensable unit which is a precursor of the cytochrome b protein that acts as a carrier in the mitochondrial electron transport chain, useful for energy production in the cells of the bird. An mtDNA cytb gene is used in molecular evolution studies and has a high power to discriminate taxonomy (Saif *et al.,* 2012; Prusak,Grzybowski & Zięba, 2004; Kuwayama & Ozawa, 2000). To justify the systematic position of the subspecies of Sarus Cranes and their genetic uniqueness at the molecular level, the present work attempts to generate relevant data and their phylogenetic structure. This study provides the first assessment of the genetic diversity of mitochondrial DNA (mtDNA) Cytochrome b (cyt b) from the Sarsai Nawar Wetland of Etawah, Uttar Pradesh along with published sequences from Genbank. The focus of this study is to; 1) establish a phylogenetic relationship among the subspecies of the Sarus Crane and 2) assess the levels of genetic diversity and differentiation among the subspecies.

Methodology

Shed feather samples were collected from the ground, in close proximity to the nests and also from confirmed territories of breeding pairs of *A. antigone* from the Sarsai Nawar Wetland (26.96223ºN, 79.23415ºE), Uttar Pradesh (Figure1).

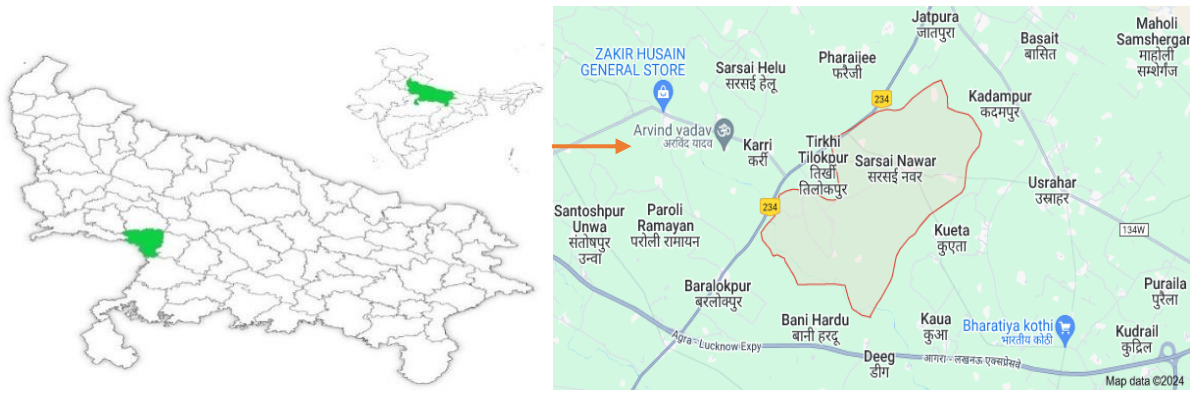

Figure 1: The Sarsai Nawar Wetland lies in the Bharthana Tehsil of Etawah District of Uttar Pradesh

The sampling localities were selected based on areas with suitable habitats that supported high densities of Sarus Cranes (Gopi Sundar 2003, Gopi Sundar & Kittur 2013, Gopi Sundar 2019). As the sex and the developmental stage of the individual bird could not be ascertained by only looking at the feather samples, all the collected feathers from one location were pooled as a single sample for analysis. Each sample was stored in a labeled plastic bag and brought to the lab at ambient temperature for later use. The calamus of good-i quality feathers was used for DNA extraction (Figure 2).

Genomic DNA was extracted using the Nucleospin Tissue XS Extraction Kit (Macherey-Nagel GmbH & Co. Germany) as per the instructions in the manufacturer's protocol. The quality and quantity of isolated DNA were analyzed following Green and Sambrook (2012).

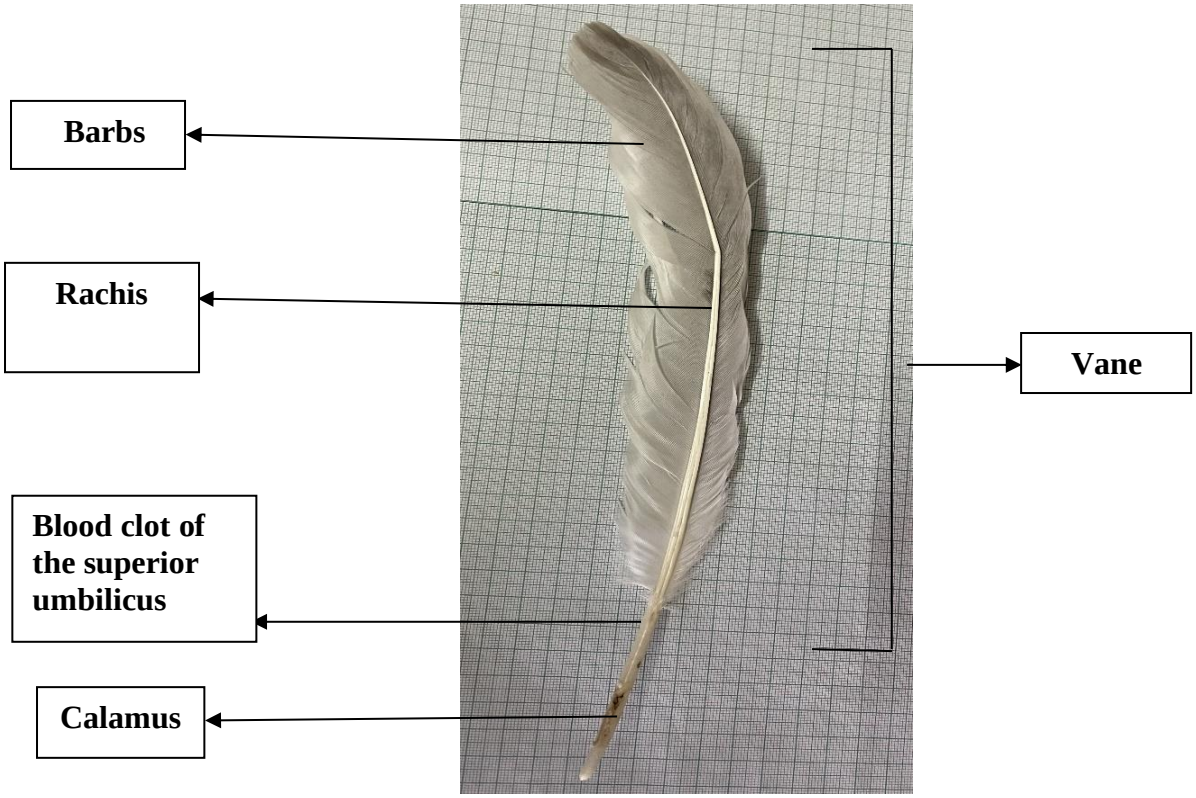

Figure 2: Parts of a shed feather from Sarus Crane. Intact calamus was used for DNA extraction from the shed feathers

Cytb gene sequences from the extracted genomic DNA were amplified by polymerase chain reaction (PCR) using primers mcb398 (5′-TACCATGAGGACAAATATCATTCTG-3′) and mcb869 (5′-CCTCCTAGTTTGTTAGGGATTGATCG-3′) (Verma & Singh 2003). Genomic DNA was amplification was carried out in reaction mixtures of 50 μl. Each reaction mixture contained 10 mM Tris-HCl (pH 8.5), 2 μ Taq polymerase, 200 μ dNTPs mix, 1.5 mM MgCl2, and 50 mM KCl 1 μl each of forward and reverse primer, with 60 ng of template DNA. An initial denaturation step at 94°C for 2 min was applied before 14 cycles of denaturation for 30 s at 94°C, hybridization for 45s at 55°C and extension for 1 min at 72°C, and 36 cycles of denaturation for 30 s at 95°C, hybridization for 45s at 51°C and extension for 1 min at 72°C followed by a final extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% agarose gel and DNA bands were visualized under a UV Trans-illuminator using a 100 bp ladder (Takara Bio) (Figure 3). All the amplified fragments were sequenced with the forward primer by the dideoxy chain termination method (Sanger's Sequencing Method) (Sanger, Nicklen & Coulson 1977).

The generated sequence of cytb gene was edited using the BioEdit program with the complete genome of *Antigone antigone* (accession number: NC_020581) as a reference and after adjusting for gaps at the beginning of the sequence with MEGA X (Tamura *et al.,* 2019). The analysis was based on a fragment of 429bp (position 588 and 1017). Phylogenetic analysis of the mtDNA haplotypes was performed using Maximum Likelihood and Neighbour Joining trees (Fatima *et.al.*2024) in MEGA X. Finally, samples from India (n = 12), Australia (n = 3) and South Eastern Asia (n = 3) were combined. The three different subspecies were considered three different populations based on previous findings of distinct morphometric and geographical locations (Jones, Barzen & Ashley, 2005). The number of haplotypes (H) haplotype diversity (h), nucleotide diversity (π) and genetic differentiation (F_{ST}) were computed to describe genetic diversity in each population using DnaSP v.6.12 (Librado & Rozas *et al.,* 2019). These included all published sequences of the species from the GenBank database (Table 3) to provide a broader understanding of how the genetic diversity of Sarus Cranes is spread across its distribution range. Connections between haplotypes were visualized by a Median-joining haplotype network created in Popart v.1.7 (Jansumat *et.al.,* 2024) (Bandelt, Forster & Rohl, 1999). Analysis of

Molecular Variance (AMOVA) and Tajima's D was computed by Arlequin v3.5 for the three subspecies populations.

Results

10 ng/µl concentration of DNA was obtained with a purity of 1.9 (260/280). Amplified sequences were visualized by gel electrophoresis where bands corresponding to 400bp to 450 bp length on the 100 bp ladder were observed (Figure 3). Upon editing the sequence a 429 bp length cytochrome b gene sequence was obtained from the shed feather of Sarus Crane from the Sarsai Nawar Wetland.

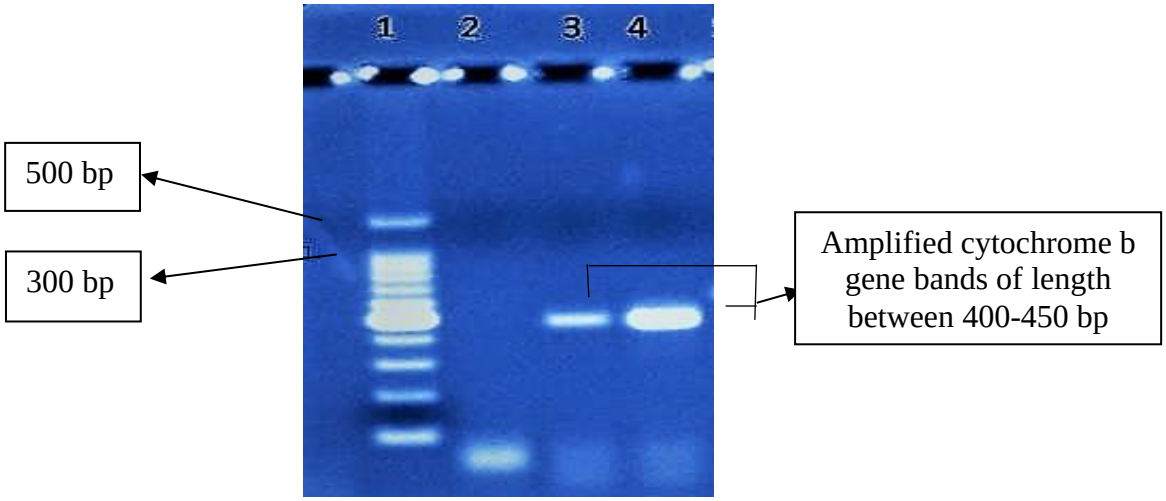

Figure 3: Gel electrophoresis plate of amplified cytochrome b gene. Lane 1: 100bp DNA Ladder. Lane 2: Negative control. Lane 3 and 4: PCR amplification of cytochrome b using Feather 1 gDNA.

The maximum likelihood tree of the sequences studied consisted of two main clades. The first clade contains two sub clades consisting of all the specimens from India and Australia, the first sub clade showed limited bootstrap support (30%) and sequence from the feather1 from the present study along with sequence with accession number KC439274 formed the second sub clade with strong bootstrap support (87%). The second clade included specimens from the Eastern subspecies only (Figure 4a) which was separated with moderate bootstrap support (61%).

Phylogenetic trees from maximum likelihood and neighbour joining methods produced congruent results consistent with the haplotype network, showing two different clades, where *Antigone a. sharpei* was the first subspecies to emerge from the mainland population and the Australian subspecies formed a single clade with its Indian counterparts. The trees are however, low resolution and do not provide sufficient information on development at the subspecies level in this sample size as each subspecies is a result of a few mutations in the core haplotypes.

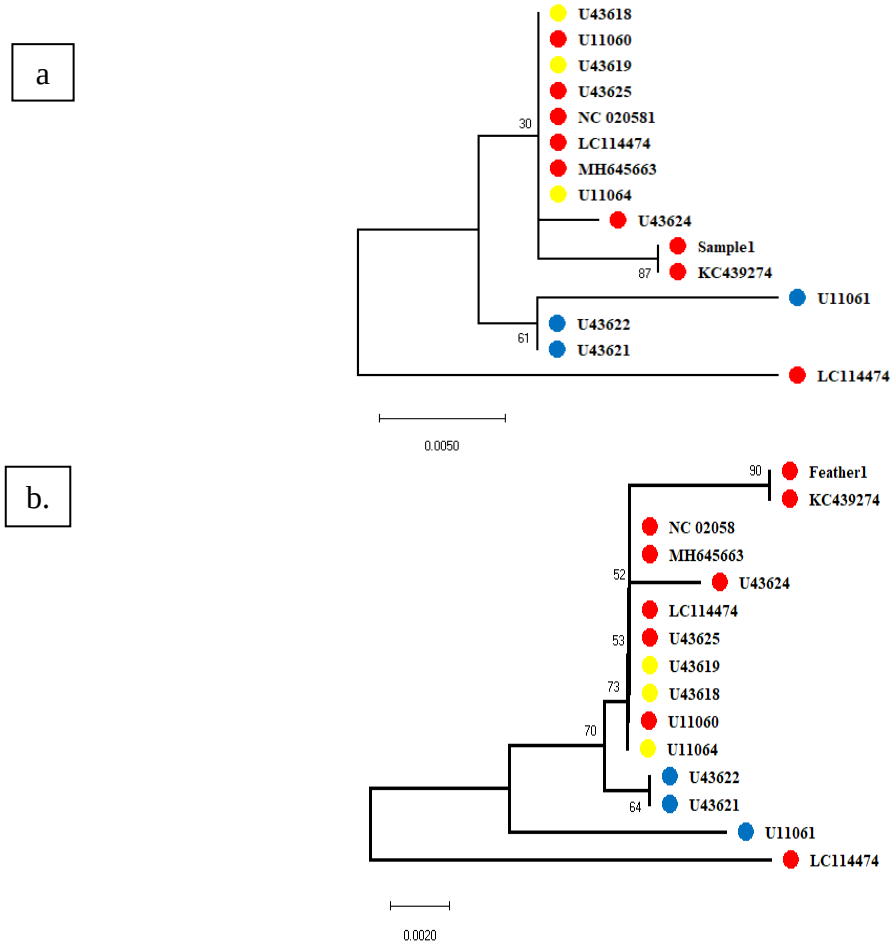

Figure 4: Phylogenetic trees of the three subspecies samples using a) Maximum Likelihood `method and b) Neighbour Joining method using MEGA X

Five distinct haplotypes were detected from the combined sample of published sequences and the sample procured in this study. The first haplotype was found in India (Hap1) and overall, in 14.2% of the samples from the study. The second haplotype was found both in Indian and Australian subspecies and was the most common haplotype (Hap2; 57%), the third and the fourth haplotypes (Hap3 and Hap4) marked their presence in one sample each from India which formed around 7% of the total population while the fifth haplotype (Hap5) was unique to the three sequences of the eastern subspecies which constituted over 21% of the total population (Table 1).

Table 1: Haplotype distribution among the three subspecies populations

S. No.	Population	No. of Samples	No. of Haplotypes	Haplotypes
1.	Indian	8	4	Hap1, Hap2, Hap3, Hap4
2.	Australian	3	1	Hap2
3.	Eastern	3	1	Hap5

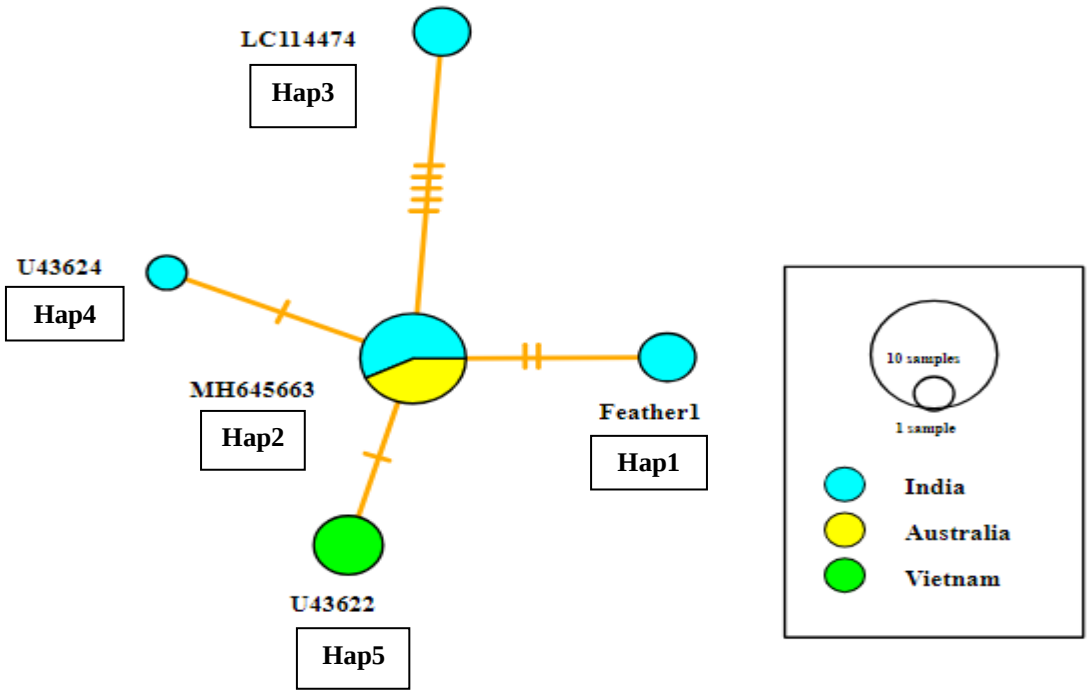

Figure 5: Haplotype Network of the sub-species samples of Sarus Cranes in the study using Popart v.1.7. Software

The Median-Joining network showed a total of nine mutational steps among all haplotypes (Figure 5). Two haplotypes (Hap1 and Hap4) were found only in the Indian subspecies. Hap1 was connected to the core haplotype (Hap2) by two mutational steps but Hap4 was a result of only one mutation. Both these haplotypes showed similar frequencies. Hap3 was separated from the core haplotype by five mutational steps and was seen in one specimen from India. The final haplotype (Hap5) was separated by one mutation from the core haplotype and was found only in specimens of the Eastern subspecies, this haplotype had the lowest frequency of all. The mean haplotype diversity was 0.695 ($h \geq 0.5$) and the average nucleotide diversity was 0.006 ($\pi < 0.5\%$). Genetic differentiation (F_{ST}) was high (0.391) between the three subspecies populations. The hierarchical AMOVA analysis of Sarus Crane populations according to their geographical location indicated that 60.83% of the total genetic variation could be attributed to genetic differences within populations and 39.16% variation was observed among the three subspecies (Table 2). The value of pairwise genetic differentiation (F_{ST}) was 0.0391. Although Tajima's D values were negative (-1.660) in samples from the three populations, they were not significant.

Table 2: Mitochondrial DNA variation within and between the Sarus Crane populations based on AMOVA

Source of variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	F_{ST}
Among populations	2	18.133	1.001	39.16570	0.39166
Within populations	12	18.667	1.556	60.83430	
TOTAL	14	36.800	2.557		

Discussion

The present study is the first population-level assessment of mtDNA cyt b diversity of Sarus Cranes. The number of cyt b haplotypes obtained was low for the overall sample. A small sample size could explain the lesser number of haplotypes we found in our sample. Despite our limited sample size, we expected to find a greater number of haplotypes. One possible explanation for the limited number of

haplotypes is a past genetic bottleneck (Lowe *et al.,* 2004). However, the limited number of haplotypes, star-like pattern of haplotypes in the median joining network, and high haplotype diversity combined with the low nucleotide diversity are consistent with a past genetic bottleneck (Lowe *et al.,* 2004). The negative value of Tajima's D (-1.660) also indicates population expansion post low frequency polymorphisms resulting from a past bottleneck. These values were however non-significant indicating that the mutations do not exert any selection pressure on the populations (Rivera-Arroyo *et. al.,* 2022). It therefore is plausible that the recent declines across their distribution have led to local reductions in the genetic diversity of Sarus Cranes. The investigated Sarus Crane sub-species populations show low average mtDNA diversity or haplotype diversity (Hd = 0.695) and nucleotide diversity (π = 0.006). This trend of high haplotype diversity and low nucleotide diversity confirms limited genetic differentiation and high frequency of private haplotypes (Rivera-Ortiz *et.al.,* 2023, Rodríguez *et. al.,* 2008). Nucleotide diversity (π) implies the average number of nucleotide differences between sequences, while haplotype diversity (Hd) indicates the probability that the haplotypes of two randomly selected individuals will differ (Nei 1987). While the AMOVA based partitioning of genetic variation in the subspecies of Sarus Cranes contributed to 39.16% of the total genetic variation among birds representing different populations, the within-population genetic diversity was 60.83%. These values are suggestive of a considerable extent of genetic structuring in the Sarus Crane subspecies based on geographical distribution. Population differentiation based on pairwise F_{ST} values recorded very high genetic differentiation among the three subspecies of the bird (F_{ST} = 0.391). As $F_{ST} \geq 0.15$ is already indicative of a significant genetic difference between subpopulations, the subspecies populations can be said to have a high level of genetic differentiation (Frankham, Ballou & Briscoe, 2002).

The findings of the present study imply that the subspecies comprise extremely different clades, which broadens our understanding of the genetic diversity of Sarus Cranes. The haplotypes were distributed in a star-shaped pattern, with the high-frequency haplotype located in the center and four low-frequency haplotypes located on the periphery, separated by only a few mutational steps. An increasing population was also suggested by this finding (Satyanarayana *et al.,* 2022).

Additional samples would be needed to establish a potential timeline for the suspected bottleneck. Overexploitation of Sarus Cranes has been observed across their distribution (Sundar 2003, Gopi Sundar 2019; IUCN 2016). Sarus Cranes in India are now threatened due to overexploitation along with the loss of critical wetland habitat and the absence of conservation measures.

Table 3: Description of cytochrome b sequences of *A. Antigone* used in the study

S.No.	Sequence label	Reference	Location	Species
1	Feather 1	Present study	India	*Antigone antigone antigone*
2	KC439274	GenBank NCBI	India	*Antigone antigone antigone*
3	MH645663	GenBank NCBI	India	*Antigone antigone antigone*
4	LC114474	GenBank NCBI	India	*Antigone antigone antigone*
5	NC_020581	GenBank NCBI	India	*Antigone antigone antigone*
6	U43625	GenBank NCBI	India	*Antigone antigone antigone*
7	U43619	GenBank NCBI	Australia	*Antigone antigone gillae*
8	U43618	GenBank NCBI	Australia	*Antigone antigone gillae*
9	U11060	GenBank NCBI	India	*Antigone antigone antigone*
10	U11064	GenBank NCBI	Australia	*Antigone antigone gillae*
11	U43624	GenBank NCBI	India	*Antigone antigone antigone*
12	U43622	GenBank NCBI	Vietnam	*Antigone antigone sharpei*
13	U43621	GenBank NCBI	Cambodia	*Antigone antigone sharpei*
14	U11061	GenBank NCBI	Vietnam	*Antigone antigone sharpei*

Despite their geographical proximity, it was observed that the Eastern subspecies consisted of haplotype which was absent in the other two populations. The Australian subspecies however, shared its haplotypes with the Indian population, these results corroborate the hypothesis that the Australian population was derived from mainland populations (Archibald, Gopi Sundar & Barzen, 2003; Jones, Barzen & Ashley 2005). Due to extensive fragmentation caused by the drastic reduction and widespread

extirpation among Asian subpopulations, these morphotypes now have reduced and non-overlapping ranges which, for Asia, are typified by very small population sizes. Since this species is threatened in all but a few areas of India (Archibald, Gopi Sundar & Barzen, 2003), conservation strategies should focus on preventing further extirpation of remnant populations, regardless of their taxonomic origin.

Our results suggest that despite the overall low mtDNA cytochrome b diversity within the Sarus Crane samples, a pattern of differentiation is present among the geographical regions, which not only holds relevance from a forensic point of view but also, the mtDNA data provides insights into genetic diversity, phylogeography, population structure and lineage tracking making it an indispensable tool in population management and conservation. These insights can potentially enable conservationists to make informed decisions to restore genetically healthy, diverse and stable populations, thereby enhancing the fitness and long-term survival of the species.

Conclusion

This study provides the first range-wide examination of mtDNA cyt b diversity in subspecies of Sarus Cranes. The samples exhibited low genetic diversity. Unique haplotypes were detected from the three subspecies that are consistent with the known postulates of taxonomic origin and patterns of gene flow in the species. The findings from phylogenetic analysis also divide the sample into two clades, where the first one comprised the Indian and the Australian subspecies while the second clade was occupied completely by the Eastern subspecies. Genetic data from the three populations exhibit a pattern expected from being subjected to a past population decline that could potentially be associated with a recent bottleneck and fragmentation of the mainland populations due to overexploitation and habitat destruction. An elaborate set of samples from all the subspecies will play a crucial role in verifying this in the future.

Conflict of Interest

There is no conflict of interest among the authors.

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