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Mitochondrial DNA analysis reveals extremely low genetic diversity in a managed population of the Critically Endangered Gharial (*Gavialis gangeticus*, Gmelin, 1789)

Surya Prasad Sharma, Suyash Katdare, Zenab Zaidi, Mirza Ghazanfarullah Ghazi, Sandeep Kumar Gupta & Syed Ainul Hussain

Wildlife Institute of India, P.O. Box # 18, Chandrabani, Dehra Dun, 248001, Uttarakhand, India

A decline in the numbers of threatened species is often reversed by reintroduction with the aim of repopulating or strengthening the population to reduce the risk of extinction. The success of reintroduction programs is associated with demographic and genetic monitoring of the reintroduced populations. We undertook a genetic assessment of the Critically Endangered gharial (*Gavialis gangeticus*) to assess the current level of genetic variation using three partial mitochondrial (mt) DNA regions: cytochrome *b*, cytochrome c oxidase subunit-I and the control region. We sequenced 103 samples collected across 14 nesting sites. A low level of mtDNA variation was observed in the sampled population (hd = 0.462 ± 0.048; Pi = 0.00029 ± 0.00004). Only five distinct haplotypes were observed in three segregating sites. This is the first assessment of the genetic variation in the wild gharial population to be made using mtDNA. Homogeneity in the 520 bp hypervariable control region of the crocodilian mtDNA is reported here for the first time. The low mitochondrial diversity and no genetic structure in the sampled population is indicative of a genetic bottleneck, the founder effect and probably associated with human-assisted augmentation of the population of the gharial. An extremely low level of genetic variation in the largest gharial population highlights the vulnerability of the gharial population in the wild and calls for immediate genetic assessment of other gharial populations so that a robust conservation plan focusing on connectivity and enhanced protection can be developed for the long-term persistence of the gharial in the wild.

Keywords: haplotype, homogeneity, low diversity, protection

INTRODUCTION

The genetic diversity of small populations is low due to various factors such as severe population declines, the founder effect and genetic bottlenecks (Banks et al., 2013). Such populations tend to lose variability rapidly as a consequence of various biotic and abiotic factors through genetic drift (Ellegren & Galtier, 2016). Drifts, regardless of any balancing force, can bring sudden and drastic changes in the allele frequency (Liao & Reed, 2009). The magnitude of such events are greater in a small population with little or no gene flow. Increased homozygosity and an increase in the frequency of recessive deleterious alleles, known as inbreeding, are immediate effects of reduced variability (Frankham et al., 1999). Long periods of isolation and inbreeding eventually lead to the decreased evolutionary adaptive potential of individuals and populations (Allendorf, 2010; Frankham et al., 1999; Galov et al., 2011; Lande et al., 1987; Liao & Reed, 2009). A decline in adaptive potential may drastically increase the extinction risk of a species locally or globally. Hence, monitoring the level of genetic variation is important for planning conservation strategies for wild and managed populations.

Species living in freshwater ecosystems are the most threatened due to natural (increasing surface temperature, non-uniform rainfall pattern) and anthropogenic (pollution, incidental capture, disturbance) factors. The gharial *Gavialis gangeticus* Gmelin (1789) is a Critically Endangered (Lang et al., 2019) freshwater crocodilian species endemic to the northern part of the Indian subcontinent (Hussain, 1999; Lang et al., 2019). The unique long-slender snout of the gharial, an adaptation for catching fish, makes it more vulnerable to accidental mortality in fishing nets (Berkovitz & Shellis, 2016; Hasan & Alam, 2016). Habitat destruction, poaching and accidental mortality in fishing gear brought the species to near-extinction. Between the 19th century and the mid-20th century, the population declined by an estimated 85 % (Hussain, 2009; Whitaker et al., 1974). Many gharial populations were extirpated in the early 1970s. By 1979, the largest known population was the one in the Chambal River, in which there were 107 individuals (all size classes) (Whitaker & Daniel, 1980).

Correspondence: Syed Ainul Hussain (hussain@wii.gov.in)

Table 1. List of primers used for the amplification of three partial mitochondrial DNA sequences

Gene	Primer	Sequence	Ta (°C)	Source
coxI	FishF1	5'-TCAACCAACCACAAAGACATTGGCAC-3'	56	Ward et al. (2005)
	FishR1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'		
cytb	CP14715	5'-TGAGGAGCAACCGTAATTACCAACCT-3'	56	Meganathan et al. (2009)
	CP15546	5'-TCTGTCTTACAAGGCCAGTGCTTT-3'		
CR	L15637	5'-GCATAACACTGAAAATGTTAAYATGG-3'	56	Oaks (2011)
	H16258	5'-CTAAAATTACAGAAAAGCCGACCC-3'		

A conservation recovery programme was initiated in the mid-1970s to avert extinction. Captive-bred and captive-reared individuals were reintroduced in the existing range, and augmentation was carried out to re-populate a suitable area with low number of gharials. In spite of tremendous conservation efforts, throughout most of its range, the gharial either failed to recover or showed extremely slow recovery rates (Nair et al., 2012).

The gharial survives in a few small, isolated populations in India and Nepal (Lang et al., 2019). The genetic diversity of the species has been considered little or not at all despite its importance in planning the conservation of threatened wildlife (Frankel, 1974). Since the ultimate goal of any conservation program is to ensure the persistence of the population in the wild, assessment of genetic diversity is essential for planning short- and long-term conservation strategies. In this study, we used three partial mitochondrial DNA (mtDNA) regions—cytochrome ***b*** (*cytb*), cytochrome c oxidase subunit-I (*coxI*) and the control region (CR)—to assess the current level of genetic variability in the largest managed gharial population.

METHODS

Study Area

The Chambal River originates in the Vindhya Hill Range in central India. It forms a part of the greater Gangetic drainage system, flowing in a north-easterly direction through the states of Madhya Pradesh, Rajasthan and Uttar Pradesh before it meets the Yamuna River. A 600 km stretch of the Chambal River between Jawahar Sagar and Panchnada was notified as National Chambal Sanctuary (NCS) in the late 1970s under Project Crocodile, for conservation of aquatic reptiles including crocodiles, freshwater turtles and the Gangetic river dolphin. Since the inception of Project Crocodile, the population in NCS has been augmented continuously. As a result of this and protection of the habitat, NCS harbours approximately 85 % of the global gharial population (Hussain, 2009).

Sample Collection

Biological sampling was conducted as part of a long-term project of ecological monitoring of the Chambal River Basin. The sampling was conducted in 14 nesting locations along the Chambal River, within NCS (Fig. 1), in 2017. We collected biological samples in the form of tissue from dead hatchlings and eggshells with the embryonic membrane intact after hatching. The samples were stored in absolute ethanol at room temperature and later, in a −20 °C in the laboratory for long-term storage. Out of 103 biological samples used in the current study, 60 samples were from obtained unique nests, 16 samples from sibling groups, and 27 samples of unknown origin.

DNA extraction, PCR and mitochondrial DNA sequencing

We carried out total genomic DNA extraction (n=103) using the Phenol–Chloroform method (Sambrook et al., 1989) with overnight digestion of embryonic membrane in a lysis buffer with Proteinase K at 56 °C.

We selected three partial mtDNA regions (*coxI*, *cytb* and CR) to assess the genetic variation in gharials. MtDNA is useful in population genetics studies because it is inherited maternally and its nucleotide substitution rate is high (Brown et al., 1979; Castro et al., 1998). The primers used to amplify partial fragments of selected regions were described by Ward et al. (2005), Meganathan et al. (2009) and Oaks (2011) respectively (Table 1). Polymerase Chain Reaction (PCR) was carried out in 20 µL volumes containing 2 µL (10−20 ng) of the DNA template, 2 µL of 10X DreamTaq buffer, 0.2 mM of each dNTP, 3 pmol of each primer and 0.1 µL (0.5 units per reaction) of DreamTaq DNA polymerase (Thermo Fisher Scientific) to amplify the fragments. The thermal profile was 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 35 seconds, 56 °C for 40 seconds and 72 °C for 45 seconds, with a final extension at 72°C for 10 minutes. The amplified products were visualised using 2 % agarose gel, and positive amplicons were cleaned up with Exonuclease-I and Shrimp Alkaline Phosphatase (USB, Cleveland, OH) and sequenced using forward primers in an Applied Biosystems 3500xL Genetic Analyzer. Standard protocols were followed when carrying out the sequencing.

Mitochondrial DNA analyses

All the gharial mtDNA sequences generated in this study were aligned using the CLUSTAL W algorithm (Thompson et al., 1994) in BioEdit, V. 7.2.6 (Hall et al., 1999). The aligned sequences and associated electropherograms were checked manually. Variations were confirmed by re-sequencing and considered only when the base Q value was greater than 20 (QV20+). The Q values were determined using Thermo Fisher Cloud (https://apps.thermofisher.com/apps/spa/#/apps). The sequences were concatenated subsequently using MEGA, V. 10.0.5 (Kumar et al., 2018). Summary statistics, including the number of haplotypes (h), haplotype (gene) diversity (hd) and nucleotide diversity (Pi), were generated for the concatenated mtDNA fragment (1800–1806 bp) using DnaSP, V. 6.12 (Rozas et al., 2017). Genealogical relationships among haplotypes were assessed using a

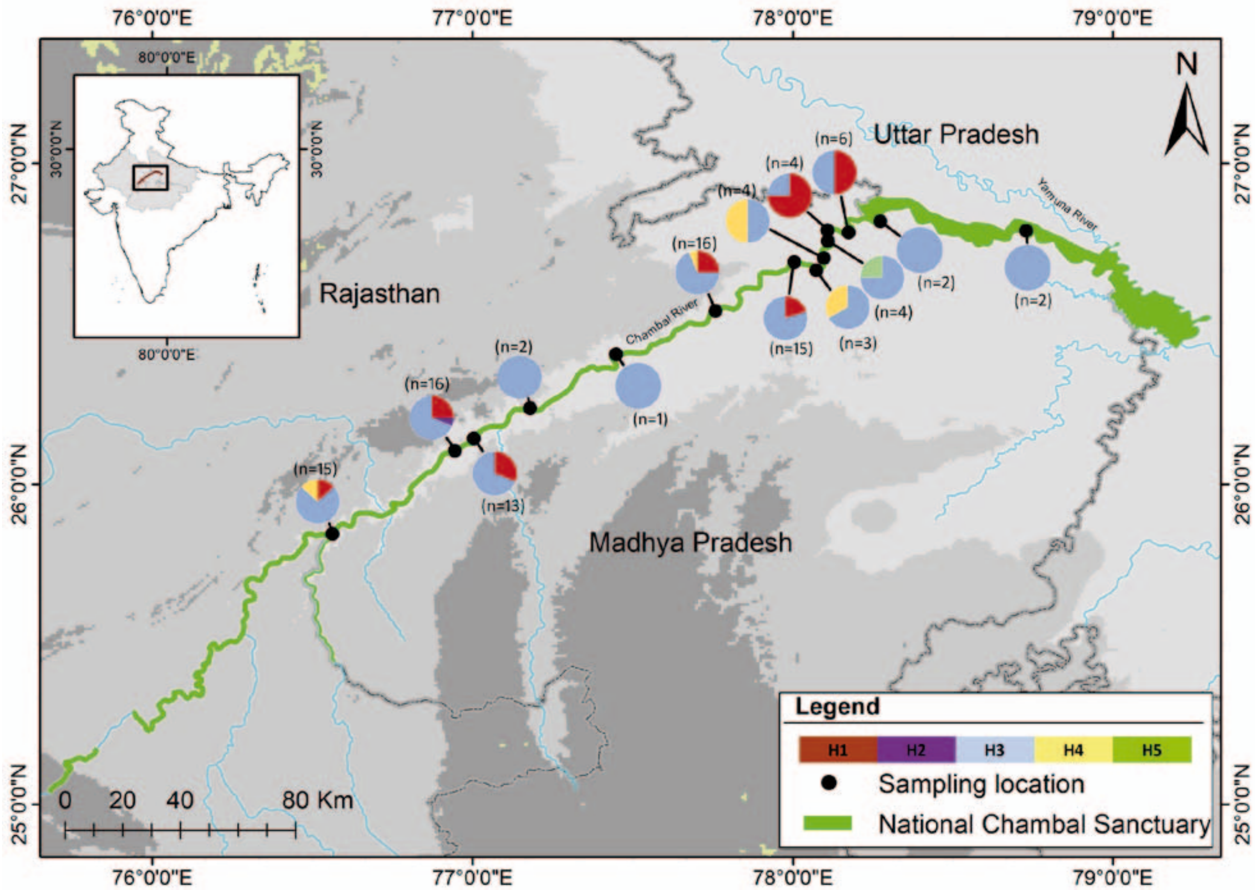

Figure 1. Map showing haplotype distribution of *G. gangeticus* in sampling locations. Pie charts represent the respective frequencies of mitochondrial DNA haplotypes in particular sampling location and n= number of samples. The colours of the pie charts represent different haplotypes.

median-joining network constructed in PopArt (Leigh & Bryant, 2015). Standard neutrality tests (*Tajima's D* and *Fu's Fs*) were performed using coalescent simulations with 10,000 permutations in Arlequin, V. 3.1 (Excoffier et al., 2005). The demographic expansion was investigated by comparison the mismatch distributions under an expected constant population and a fluctuating population with 10,000 coalescent simulations using DnaSP, V. 6.12 (Rozas et al., 2017).

RESULTS

Mitochondrial DNA diversity

1,798 – 1,806 bp of sequence data (609 bp *coxI*, 673–676 bp *cytb* and 488–521 bp CR) was obtained from 103 individuals. We observed five distinct haplotypes (H1–H5), with three segregating sites (one singleton and two parsimony informative sites) (Table 2). The sequences were submitted to GenBank (Supplementary Table S1). The haplotype (gene) diversity (hd, mean ± SD) was 0.462 ± 0.048, and the nucleotide diversity (Pi, mean ± SD) was 0.00029 ± 0.00004. Because there was no variable site in the CR, the diversity was not calculated for separate gene sequences.

The haplotype network showed no genetic cluster among the nesting localities (Figs. 1 and 2). The network was resolved with three major haplotypes. Most of the haplotypes differed from each other by one or two nucleotides. The haplotype H3 was the most common haplotype, and it was found in 72 samples (69.9 %) distributed among all the nesting locations. H1 contained 23 samples (22.3 %) from seven nesting localities, and H4 was found in six samples (5.8 %) from four nesting localities. H2 and H5, each contained one sample (1 %) each.

The mismatch distribution curve appeared to be unimodal. The standard neutrality test for population stability *Tajima's D* = -0.21 (*p*-value 0.45) and *Fu's Fs* = -1.394 (p-value 0.23) yielded a negative non-significant value.

DISCUSSION

This study represents the first genetic assessment of the wild gharial population using mtDNA. The study reveals that the variability in the 1,806 bp of mtDNA analysed here is low. The low level of variation in the mtDNA sequences is concordant with reports of low levels of mtDNA variation reported in other crocodilian species (Bloor et al., 2015; Glen et al., 2002; Luck et al., 2012; Posso-Pelaez et al., 2018; Ray et al., 2004). However, the homogeneity of the hypervariable control region is unusual. This is the first study to report homogeneity in a 520 bp control region in crocodilians. The low levels of haplotype diversity and nucleotide diversity observed in the sampling localities are possibly explained by the

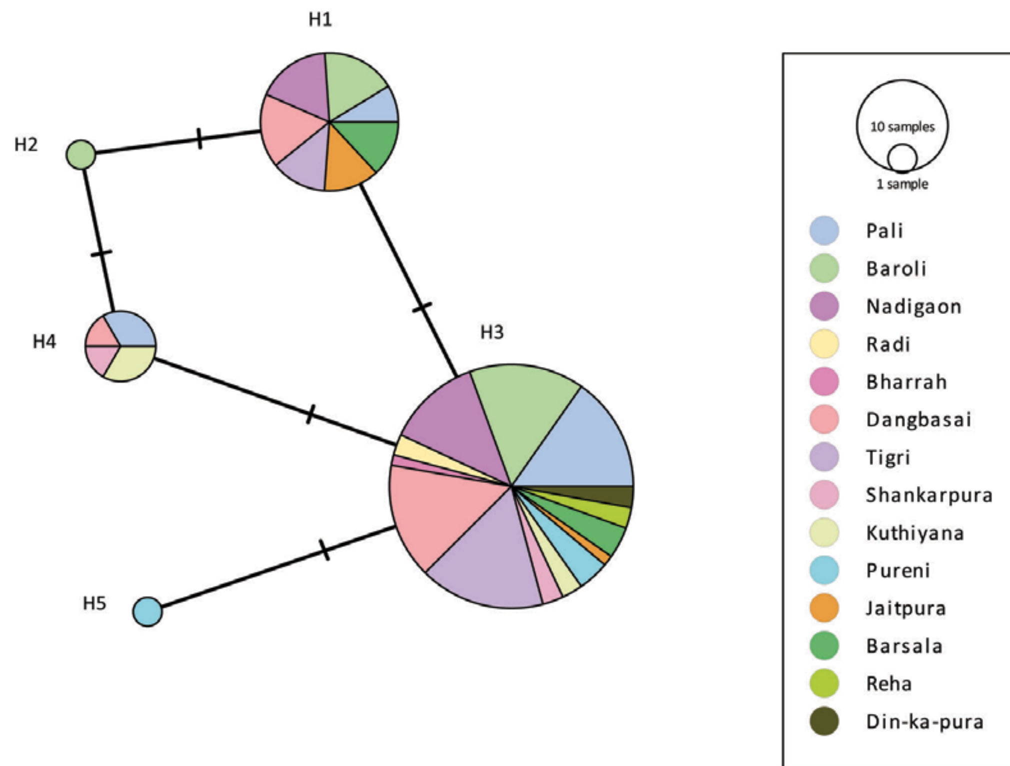

Figure 2. Median-joining haplotype network inferred from 1,806 bp of mtDNA sequence data for *G. gangeticus*. Each circle represents a different haplotype and the size is indicative of number of individuals present within the haplotype. The colours represent the proportions of the haplotypes from each nesting locality.

Table 2. Variable nucleotide position for five haplotypes observed using the concatenated approach.

Halotype number	Nucleotide position 108	Nucleotide position 703	Nucleotide position 1182
H1	T	G	C
H2	.	.	T
H3	C	.	.
H4	C	.	T
H5	C	A	T

known history of a severe population decline, genetic bottleneck and assisted population recovery with a small number of founder individuals.

The genealogical relationship determined using the median-Joining network, with no distinct clusters in the nesting localities, suggests that there is a high degree of haplotype sharing. This could be attributed to the continuous and unstructured release of young gharials that has taken place in the upstream sections of the river. The non-significant negative value obtained from the standard neutrality tests (*Tajima's D* and *Fu's Fs*) serves as a caution against drawing inferences. The unimodal mismatch distribution curve is indicative of a population expansion. However, the time of occurrence and the magnitude of the event remain unknown.

Further study needs to be carried out using nuclear microsatellite markers, single nucleotide polymorphism or other high-throughput molecular tools to substantiate the findings of our study and to gather evidence about the time and impact of demographic events. Genetic analyses of samples from other gharial populations are critical in order to establish the possible reason for the low mtDNA variation observed in this study and to develop a robust conservation strategy.

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