

**GENETIC DIVERSITY, GENE-FLOW AND MATING SYSTEM
IN SYMPATRIC CROCODYLIAN SPECIES IN NATIONAL
CHAMBAL SANCTUARY, INDIA**

Thesis submitted to the
Saurashtra University,
Rajkot (Gujarat)



For the award of the Degree of
DOCTOR OF PHILOSOPHY
IN
WILDLIFE SCIENCE

By
SURYA PRASAD SHARMA

Registration No: 17240

WILDLIFE INSTITUTE OF INDIA



February 2023

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Under the Supervision of

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Dr. S. A. HUSSAIN

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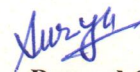


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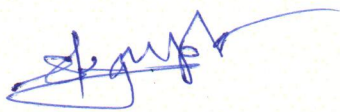

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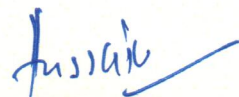
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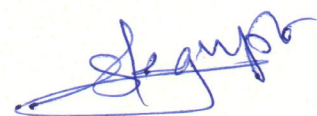
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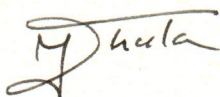
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Mr. Surya Prasad Sharma has put in more than six terms of research work embodied in this thesis under our guidance and supervision. The work presented in this thesis has not been submitted for any degree of any other University or Institution.


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TABLE OF CONTENTS

	<i>Page No.</i>
List of Figures	iv
List of Tables	viii
List of publications	ix
Acknowledgement	xii
Executive Summary	xv
Chapter 1: Introduction	
1.1 Background	1
1.2 Origin and evolution of Crocodylia	2
1.3 Systematics of the extant crocodylians	4
1.4 Geographical distribution	8
1.5 Threats	9
1.6 Study species	11
1.6.1 Gharial (<i>Gavialis gangeticus</i>)	11
1.6.2 Mugger (<i>Crocodylus palustris</i>)	13
1.7 Crocodylian conservation in India	14
1.8 Role of Conservation genetics in species recovery	15
1.9 Significance of the study	19
1.10 Literature review	21
1.10.1 Crocodile conservation in the past	21
1.10.2. Preservation of genetic diversity	24
1.11 Objectives and research questions	26
1.12 Hypothesis	27
1.13 Study Area	28
1.13.1 National Chambal Sanctuary (in Chambal River)	28
1.13.2 Katarniaghat Wildlife Sanctuary (in Girwa River)	30
1.14 Organisation of the thesis	31

Chapter 2: Genetic variation among the gharial and mugger populations using mitochondrial and nuclear DNA markers

2.1 Summary	32
2.2 Introduction	33
2.3 Methodology	38
2.3.1 Sample collection	38
2.3.2 DNA extraction	40
2.3.3 Selection and screening of markers	40
2.3.4 Genotyping quality	44
2.3.5 Mitochondrial DNA variation	45
2.3.6 Nuclear microsatellite variation	46
2.4 Results	46
2.4.1 Mitochondrial DNA variation	47
2.4.2 Genotyping quality	51
2.4.3 Nuclear microsatellite variation	52
2.5 Discussion	55

Chapter 3: Population genetic structure, differentiation, and gene-flow

3.1 Summary	59
3.2 Introduction	60
3.3 Methodology	63
3.3.1 Sample collection	63
3.3.2 DNA extraction	66
3.3.3 Genotyping quality	64
3.3.4 Nuclear microsatellite variation	65
3.3.5 Population genetics structure and Genetic differentiation	65
3.3.6 Gene-flow	66
3.4 Results	67
3.4.1 Genotyping quality and error rates	67

3.4.2 Nuclear microsatellite variation	69
3.4.3 Population genetic structure and genetic differentiation	71
3.4.4 Gene-flow	80
3.5 Discussion	83
Chapter 4: Incidence of multiple paternity in gharial and mugger	
4.1 Summary	89
4.2 Introduction	90
4.3 Methodology	93
4.3.1 Sample collection and DNA extraction	93
4.3.2 Microsatellite selection and effectiveness of the markers	93
4.3.3 Analysis of multiple paternity	94
4.4 Results	95
4.5 Discussion	100
Chapter 5: Summary	
5.1 Summary	103
5.2 Conservation Implication	107
References	111
Appendices	141

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LIST OF FIGURES

Figure No.	Title	Page No.
Figure 1.1	Phylogeny of Archosauria clade based on current consensus of their inter-relationships (Bishop <i>et al.</i> , 2020).	2
Figure 1.2	A species tree representation of all extant Crocodylians. The dashed lines indicate discordance between morphological and molecular datasets. Morphological traits suggest the inclusion of Tomistoma in the Crocodyloidea clade, however, molecular traits point towards its inclusion into the Gavialoidea clade (Grigg., 2015)	5
Figure 1.3	Crocodylia cladogram based on morphological dataset illustrating the extant crocodylian interrelationships (Rio and Mannion 2021).	6
Figure 1.4	Map showing the extant distribution of gharial (<i>Gavialis gangeticus</i>), mugger (<i>Crocodylus palustris</i>) and saltwater crocodile (<i>Crocodylus porosus</i>).	10
Figure 1.5	Map showing National Chambal Sanctuary (orange color) within Chambal River. A ~600km of the Chambal River is protected for the conservation of the aquatic fauna.	29
Figure 1.6	Map showing Katarniaghat Wildlife Sanctuary (dashed line). A 20km stretch of Girwa River within the Katarniaghat Wildlife Sanctuary is prime habitat for gharial.	30
Figure 2.1	Detailed representation of gharial and mugger samples collected for genetic assessment along the Chambal River.	40
Figure 2.2	Graphical representation of haplotype diversity (hd) estimated using two partial mitochondrial fragments	47

Figure No.	Title	Page No.
	(<i>cytochrome b</i> and control region) in gharial (<i>Gavialis gangeticus</i>) and mugger (<i>Crocodylus palustris</i>).	
Figure 2.3	Graphical representation of nucleotide diversity (π) estimated using two partial mitochondrial regions (<i>cytochrome b</i> and control region) in gharial (<i>Gavialis gangeticus</i>) and mugger (<i>Crocodylus palustris</i>).	48
Figure 2.4	Graphical representation of nucleotide diversity (π) estimated across 23 crocodylian species.	50
Figure 3.1	Population genetic structure of 228 gharial individuals derived using a Bayesian clustering approach implemented in STRUCTURE without location priors. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster.	72
Figure 3.2	Population genetic structure of 228 gharial individuals derived using a Bayesian clustering approach implemented in STRUCTURE with location priors. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster.	73
Figure 3.3	Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 228 gharial individuals. This scatterplot shows the first two principal components of the DAPC. The individuals are shown as point.	74
Figure: 3.4	Population genetic structure of 67 mugger individuals derived using a Bayesian clustering approach implemented in STRUCTURE without location priors. A single bar plot represents the individual, and the extent of	75

Figure No.	Title	Page No.
	colour in each bar indicates the probability of assigning the individual to a particular cluster.	
Figure 3.5	Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 67 gharial individuals. This scatterplot shows the first two principal components of the DAPC. Dots represent individuals.	76
Figure 3.6	Population genetic structure of 59 mugger individuals derived using a Bayesian clustering approach implemented in STRUCTURE without location priors. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster.	77
Figure 3.7	Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 59 mugger individuals. This scatterplot shows the first two principal components of the DAPC. Dots represent individuals.	78
Figure 3.8	Recent migration rate (m) estimated in gharial individuals between Girwa and Chambal population estimated using a Bayesian approach implemented in BayesAss. The values in the parenthesis represents 95% confidence interval. CH=Chambal and GR=Girwa River.	81
Figure 3.9	Recent migration rate (m) estimated in mugger individuals between Girwa and Chambal population using Bayesian approach implemented in BayesAss. The values in the parenthesis represents 95% confidence interval. CH=Chambal and GR=Girwa River.	82
Figure 3.10	Recent migration rate (m) estimated within Chambal River mugger individuals using Bayesian approach implemented	83

Figure No.	Title	Page No.
	in BayesAss. The values in the parenthesis represents 95% confidence interval. PA=Pali, BR=Baroli, NG-Nadigaon and TG=Tigri.	
Figure 4.1	Probability of detecting multiple paternity (PrDM) for five microsatellite loci in mugger at different number of putative males (sire ratio) and number of offspring per clutch.	98

LIST OF TABLES

Table No.	Title	Page No.
Table 1.1	List of recognized crocodylian species and their IUCN status (IUCN-CSG, Oaks 2011). LC- Least concern, CR- Critically Endangered, VU- Vulnerable.	7
Table 2.1	Molecular genetics utility of different mitochondrial genes/regions. The size of asterisk signifies the useability of the region/gene in each category.	42
Table 2.2	Primer sequence used for the amplification of partial mitochondrial <i>cytb</i> and control region.	43
Table 2.3	Detail of gharial and mugger samples collected for genetic diversity estimation in the Chambal River (T=Tissue and M=chorioallantoic membrane).	46
Table 2.4	A comparative chart of genetic diversity estimated using 72 complete mitochondrial genomes of 23 recognized crocodylian species. Number of sequences (N), the total number of sites (S), polymorphic or variable sites (P), the number of haplotypes (h), haplotype diversity (hd) and nucleotide diversity (Pi).	49
Table 2.5	Amplification success and error rates calculated for seven polymorphic loci in gharial samples.	51
Table 2.6	Amplification success and error rates calculated for seven polymorphic loci in gharial samples.	52
Table 2.7	Genetic diversity estimates of 162 individuals of gharial at seven microsatellite nuclear loci. The number of individuals (N), number of alleles per locus (Na), allelic richness (Ar), observed heterozygosity (Ho), expected	53

Table No.	Title	Page No.
	heterozygosity (He), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS).	
Table 2.8	Genetic diversity estimates of 60 individuals of mugger at ten microsatellite nuclear loci. The Number of individuals (N), number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS), not done (ND), * significant at $P<0.05$.	54
Table 3.1	Detail of gharial and mugger samples collected for population genetics structure and gene-flow (T=Tissue and M=chorioallantoic membrane)	64
Table 3.2	Summary of the amplification success and error rates frequency calculated for gharial samples genotyped at seven polymorphic loci	67
Table 3.3	Summary of the amplification success and error rates frequency calculated for mugger samples genotyped at ten polymorphic loci	68
Table 3.4	Genetic diversity estimates of 66 gharial individuals (Girwa River) six polymorphic microsatellite nuclear loci. Number of individuals (N), number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS), not done (ND).	70
Table 3.5	Genetic diversity estimates of eight individuals (Girwa River) of mugger at ten microsatellite nuclear loci.	70

Table No.	Title	Page No.
	Number of individuals (N), number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS), not done (ND).	
Table 3.6	Number and percent of gharial individuals assigned to each genetic cluster inferred using Bayesian clustering approach implemented in STRUCTURE	72
Table 3.7	Number and percent of gharial individuals assigned to each genetic cluster inferred using Bayesian clustering approach implemented in STRUCTURE	73
Table 3.8	Number and percent of mugger individuals assigned to each genetic cluster inferred using Bayesian clustering approach implemented in STRUCTURE	75
Table 3.9	Number and percent of mugger individuals assigned to each genetic cluster inferred using bayesian clustering approach. PA-Pali, BR-Baroli, NG-Nadigaon and TG-Tigri	79
Table 3.10	Genetic differentiation between nesting sites in Chambal River. The value below diagonal represents F_{ST} and below represents p-value. *= represents significant p-value. PA-Pali, BR-Baroli, NG-Nadigaon and TG-Tigri	79
Table 3.11	Genetic differentiation between nesting sites in Chambal River. The value below diagonal represents G'_{ST} and below represents p-value. *= represents significant p-value. PA- Pali, BR-Baroli, NG-Nadigaon and TG-Tigri	80
Table 3.12	Genetic differentiation between nesting sites in Chambal River. The value below diagonal represents Jost's D and	80

Table No.	Title	Page No.
	below represents p-value. *= represents significant p-value. PA- Pali, BR-Baroli, NG-Nadigaon and TG-Tigri	
Table 4.1	List of species-specific and cross-species polymorphic microsatellite loci in gharial and mugger. * Loci selected for paternity analysis in mugger	95
Table 4.2	Combined exclusion probability estimated to assess the effectiveness of microsatellite loci to perform parentage analysis in mugger. The exclusion probability was estimated for complete set of loci.	97
Table 4.3	Combined exclusion probability estimated to assess the effectiveness of microsatellite loci to perform parentage analysis in gharial.	97
Table 4.4	Number of alleles per locus per nest. MF=Minimum number of fathers inferred using multilocus stepwise progeny arrays method	99

EXECUTIVE SUMMARY

The order Crocodylia is represented by 27 species and three families: Alligatoridae, Crocodylidae and Gavialidae. India harbours three crocodylian species: the gharial (*Gavialis gangeticus*), the mugger or marsh crocodile (*Crocodylus palustris*), and the saltwater crocodile (*Crocodylus porosus*). Over the years, the crocodylian population has suffered a population decline and substantial range contraction due to habitat loss, poaching, and mortalities in passive fishing. The population in India has recovered from an extreme low in numbers through a conservation effort ‘Project Crocodile’ initiated in the mid-1970s. The primary strategy of the program was assisted conservation translocation through ‘head-starting of individuals’ to achieve quick recovery of the species in its respective distribution range. In head -start approach wild collected eggs were hatched and reared in rehabilitation centres, and individuals after attaining a length of 1.2 m were translocated into suitable habitats within the newly created protected areas. Both the freshwater crocodylians viz. gharial (*Gavialis gangeticus*) and mugger (*Crocodylus palustris*) have increased in number since the initiation of the Project Crocodile and are now regarded as recovered. This recovery, however, has largely been based demographic monitoring mainly change in abundance without considering important criteria such as behavioural, genetic, health and mortality and social and economic monitoring essential for measuring the efficacy of such programs.

Genetic diversity is important for the long-term viability of species, as it provides the genetic variation necessary for adaptation to changing environments and resilience to

diseases and other threats. The usefulness of genetic information is unrivalled in species that have recovered from severe demographic decline and occurring in small and isolated populations. Genetic monitoring can also provide comprehensive information on the recovery of the population at risk. Unlike population monitoring that become a routine practice and gained acceptance among the scientific community, managers, and policymakers for monitoring the wild and measuring the recovery of the translocated populations. The usability and inclusion of genetic monitoring for spatial and temporal change in genetic diversity is still not commonplace, largely, due to its complex nature, lack of guidelines, and their direct significance. Thus, despite its recognized role, genetics is often overlooked in monitoring the recovery of the translocated population. The present research was, thus, undertaken to provide information on the genetic status of two freshwater crocodylian species through assessment of the (a) genetic diversity, (b) population genetic structure, genetic differentiation, and gene-flow and (c) mating system using nuclear microsatellite and mitochondrial markers. The biological samples for genetic assessment were collected from two of the largest breeding gharial population in Chambal River (within the National Chambal Sanctuary) and Girwa River (within Katarniaghat Wildlife Sanctuary) for both the study species.

For genetic diversity assessment, a total of 232 gharial and 81 mugger samples collected from Chambal River were successfully genotyped at seven and ten polymorphic loci, respectively. 162 (71%) unique gharial individuals and 60 (74%) unique mugger individuals were identified using multilocus genotype data. The number of alleles per locus ranged was higher in mugger samples (Gharial – 2 to 7 and mugger – 2 to 14). The

heterozygosities were also higher in mugger samples, the observed (H_o) and expected (H_e) heterozygosities were 0.42 ± 0.07 and 0.41 ± 0.06 in gharial and $H_o = 0.58 \pm 0.09$; $H_e 0.55 \pm 0.08$ in mugger. A low level of mitochondrial DNA variation in the both the species. The mitochondrial control region (CR) sequences ($n=103$) in gharial were invariable with a single haplotype and the mugger CR sequences ($n=51$) had two haplotypes (mean $\pm$ SD) ($h_d = 0.039 \pm 0.037$; $\pi = 0.00007 \pm 0.00007$). The gharial Cytochrome b sequences ($n=103$) revealed three haplotypes ($h_d = 0.146 \pm 0.00207$; $\pi = 0.00022 \pm 0.00007$) and mugger sequences ($n=60$) exhibits two haplotypes ($h_d = 0.066 \pm 0.043$; $\pi = 0.00012 \pm 0.00008$)

Additionally, 116 gharial and 16 mugger samples from Girwa River were used to derive the population genetic structure, genetic differentiation, and gene-flow. No clear signature of a genetic cluster was observed in gharial, whereas low but distinct level of genetic differentiation with geographical segregation was observed in gharial samples. No intra-population genetic structure was observed in gharial samples. The gene-flow between the populations (Chambal and Girwa) was measured and the recent migration rate (m) was 0.12 (95% CI 0–0.26) from Chambal to Girwa River and 0.02 (95% CI 0–0.05) from Girwa to Chambal. The mugger showed a presence of population genetic structure at both intra-population (within Chambal River) and inter-population (Chambal and Girwa river) samples and supported by significant level of genetic differentiation. The gene-flow between the populations (Chambal and Girwa) measured as the recent migration rate (m) was 0.035 (95% CI 0–0.096) from Chambal to Girwa River and 0.007 (95% CI 0–0.0019) from Girwa to Chambal.

Multiple paternity in the mugger samples was in one of the two nest clutches collected from the Nadigaon nesting site in the Chambal River using single locus minimum (manual method) and multilocus stepwise progeny arrays method. The multilocus stepwise progeny arrays method confirmed the contribution of minimum of two males. Multiple paternity in crocodylians is a well-known phenomenon occurring in up to 95 % instances, often with a contribution of up to five males in single clutch of eggs. The paternity analysis provides multiple benefits such as fertilization assurance, additional partner to care for hatchlings and genetic benefits. Multiple paternity in species is viewed as evolutionary and or behavioural mechanism linked with increased reproductive success, genetic fitness, and social benefits. Extra-pair paternity (polyandry) is known to increase the effective population size, increased genetic variation and evolutionary potential in species. The incidence of multiple paternity could not be determined in gharial samples due to low statistical power (combined exclusion probability and probability of detecting multiple paternity) obtained using seven polymorphic nuclear microsatellites loci.

Overall, the genetic diversity observed at seven polymorphic nuclear microsatellite loci was lower in the gharial than mugger population. The low genetic diversity and indicative of a genetic bottleneck, the founder effect and probably associated with human assisted corresponds with species life-history traits. Higher proportion of admixed gharial individuals and relatively high gene-flow indicate human mediated gene-flow in gharial, while presence of intra-and inter population structure supported by estimates of genetic differentiation in mugger is likely due to naturally growing mugger population. The

presence of multiple paternity in mugger indicates presence of polyandry in mugger population.

The low genetic diversity in gharials is a significant conservation challenge that must be addressed to ensure the long-term survival of the species in the wild. This essentially requires continued implementation of adaptive conservation strategies such as increase monitoring of focal species and co-occurring species along with the ongoing conservation translocation program. Moreover, the conservation of the crocodylians in is a multi-faceted issue that requires a combination of regulatory measures and habitat protection. The crocodylian in Inidan and across the globe are currently facing numerous anthropogenic threats, including climate change. Rising temperatures, changing rainfall patterns, and sea level rise are directly or indirectly affecting species, populations, and their habitats. These impacts of likely to become evident and widespread due to climate change, as they are highly dependent on temperature regimes for regulating their body temperature and for controlling their reproductive cycles. Changes in temperature, declining river water level and rainfall patterns can cause fluctuations in food availability and are likely to reduce the available habitat for the species.

To address these threats, conservation efforts must focus on protecting and restoring habitats, reducing human-crocodile conflict, and monitoring crocodile populations to detect any declines. This can be achieved through a combination of measures, such as the creation of conservation priority areas, the restoration of degraded (sub-optimum) habitats, and the promotion of sustainable use practices.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The role of large-bodied predators in their respective ecosystems has proven to be crucial for maintaining various ecological processes (Somaweera et al., 2020). Despite the critical importance of large predators for maintaining ecosystem processes, they are highly threatened and declining at an alarming rate due to the combined effect of climate change and anthropogenic stressors. The decline in their number might cease to perform crucial ecosystem functioning well before they go extinct (Valiente-Banuet et al., 2015). Species occurring in aquatic ecosystems, whether freshwater or marine, are exceedingly threatened due to these factors (Strayer & Dudgeon, 2010).

Crocodylians are large body semi-aquatic (freshwater and marine) predators that serve the role of a top predator in the ecosystem they occur (Mazzotti et al., 2009; Somaweera et al., 2020). The crocodylians, pervasive in much of their geographic distribution, have suffered a dramatic decline in their number and contracted in distribution range in the last century (Whitaker & Andrews, 2003). Habitat loss, poaching, killing for hides, and passive mortality in fishing gear were common causes of the decline of crocodylians across the globe (Hussain, 1999; Whitaker & Andrews, 2003). Crocodylian conservation has gained attention since the mid-20th century, with efforts geared toward protecting these species through a range of activities such as designating suitable habitats as Protected Areas (PAs) – to provide species adequate space for survival, conservation translocation – to boost the population for extremely

depleted species and to provide species legal protection – limit the illegal trade of species. Similarly, crocodile conservation in India and around the world has received much attention after the end of World War II (Singh 1978, 1982)

1.2 ORIGIN AND EVOLUTION OF CROCODYLIA

The Archosauria, or ‘*ruling reptile*’ is the clade that includes extant crocodylians, aves, and other extinct groups, including dinosaurs (Brocklehurst et al., 2020; Brusatte et al., 2010). The member of clade archosaurs originated approximately 250 million years ago (mya) along with the ancestors of modern lissamphibians, turtles, lizards, and mammals shortly after the Permian-Triassic extinction (Benton, 2014; Bishop et al., 2020; Nesbitt, 2011) (Figure 1.1).

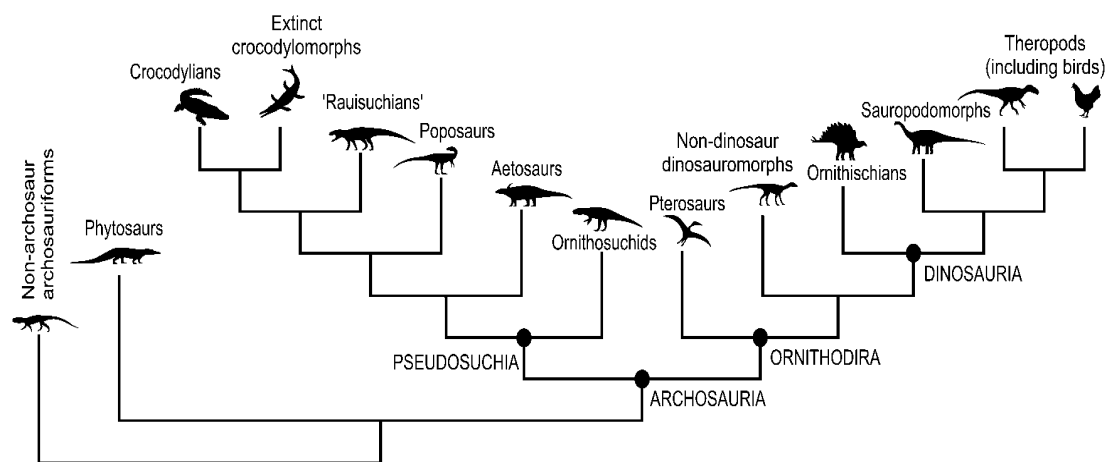


Figure 1.1: Phylogeny of Archosauria clade based on the current consensus of their inter-relationships (Bishop et al., 2020).

The clade archosaurs diversified during the Triassic period and gave rise to *Ornithodira* and *Pseudosuchia* lineages. The *Ornithodira* includes extant avian and

extinct dinosaurs, and Pseudosuchia includes extant crocodylians and extinct members of crocodylomorphs (Bishop et al., 2020).

The ancestors of the modern crocodylians, which were similar in form and function to the extant crocodylians, originated during the late Triassic within larger clade crocodylomorphs. Since the Triassic, most of the crocodylians that emerged were semi-aquatic, including some forms that appeared during the Jurassic, and were highly adapted to marine life (Benton, 2014). The first crocodylian-like members, such as *Protosuchus* and *Orthosuchus*, appeared in the early Jurassic and displayed various characters of crown crocodylian synapomorphies (Benton, 2014; Grigg & Kirshner, 2015). The true or proper crocodylian clade Eusuchia are descendent of Mesoeucrocodylia, and their origin extends back to the Cretaceous–Palaeogene extinction event, which happened approximately 66 mya that wiped many crocodylians and non-avian dinosaurs. The surviving members of this clade diversified later during the Mesozoic and Palaeogene, especially during periods of higher global temperatures, paralleled by expansion and contraction in geographical ranges (Brochu, 2003; Brochu et al., 2022; Groh et al., 2020). The crocodylomorph clade was highly diversified during the Jurassic – Cretaceous and may have even reached ~300 species (Godoy et al., 2019).

The study of species evolution, the evolutionary relationship between species, the progression of evolution, and numerous species-specific evolutionary theories are based on fossil evidence (Brochu & Sumrall, 2020). The lack of supporting fossil evidence limits the understanding of theories and progression. Hence, theories and evolutionary progressions are inherently unstable and prone to change in response to

discovering new fossil links (Benton, 2014; Brochu et al., 2022; Nesbitt, 2011). Similarly, the understanding of the evolutionary history of crocodylians is likewise unresolved due to the absence of fossil links (Bronzati et al., 2015; Cidade et al., 2019). For instance, fossil evidence suggests the ancestors of the obligate freshwater crocodile ‘gharial’ lived in saltwater or could withstand saline conditions (Brochu et al., 2007; Vélez-Juarbe et al., 2007). The lack of “transition fossils” supporting the marine-freshwater transition of the freshwater gharial, the likely origin, evolutionary progression, and systematic relationship are highly debated (Iijima et al., 2016, 2022).

1.3 SYSTEMATICS OF THE EXTANT CROCODYLIANS

The order Crocodylia, an ecologically diverse clade, is distributed throughout the tropical and sub-tropical regions in Asia, Australia, Africa, and North and South America, except for the Arctic and Antarctica. Currently, the clade is represented by 27 species in nine genera (Grigg & Kirshner, 2015; Hekkala et al., 2011; Shirley et al., 2014, 2018) (Table 1.1).

Like other taxa, the systematics of the order Crocodylia have been debated due to discordant findings on the comparison of molecular and morphological datasets. Hence, recent studies on crocodylian systematics have been driven to resolve the incongruities between molecular and morphological data (Oaks, 2011). The major disparity in crocodylian systematics was about the placement of *Gavialis gangeticus* and *Tomistoma schlegelii* (Figure 1.2) and the revision of family-level systematics to recognize the existence of two families *viz.* Alligatoridae and Crocodylidae (based on molecular studies) instead of the three families Alligatoridae, Crocodylidae, and

Gavialidae recognized earlier, wherein the members of earlier recognized family Gavialidae (*Gavialis gangeticus* and *Tomistoma schlegelii*) are placed in Crocodylidae under subfamily Gavialinae (Grigg & Kirshner, 2015; Janke et al., 2005; Oaks, 2011) (Figure 1.2).

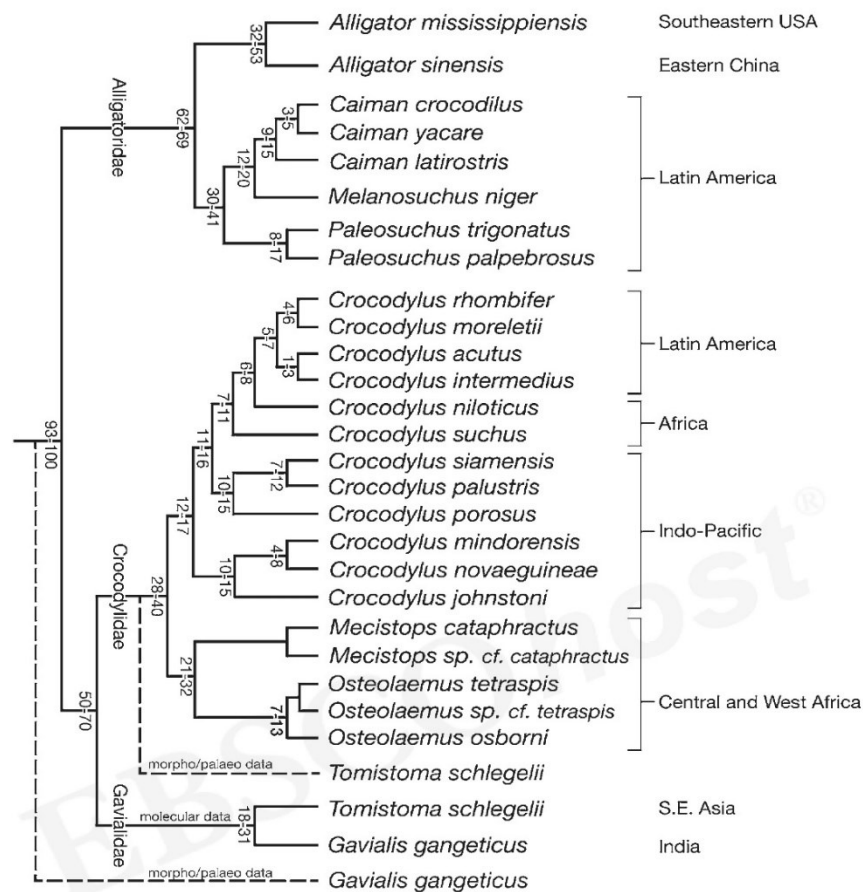


Figure 1.2: A species tree representation of all extant Crocodylians. The dashed lines indicate discordance between morphological and molecular datasets. Morphological traits suggest the inclusion of *Tomistoma* in the Crocodylidae clade. However, molecular features point towards its inclusion into the Gavialidae clade (Grigg & Kirshner, 2015).

Further recognition of new species and revision of species-level phylogeny is in progress within the order of Crocodylia. Studies based on the consensus of

morphological and molecular datasets have identified 24 species in three families: Alligatoridae, Crocodylidae, and Gavialidae (Table 1.1). However, recent systematic studies utilizing advanced algorithms, extensive molecular datasets, and morphological data suggested the presence of 25 Crocodylian species (Hekkala et al., 2011; Oaks, 2011) and 27 species (Grigg & Kirshner, 2015; Shirley et al., 2018) (Figure 1.2).

Until recently, gharial (*Gavialis gangeticus*) was considered the basal and only extant member of the family Gavialidae (Rio & Mannion, 2021)(Figure 1.2). However, phylogenetic analyses based on molecular data suggest Alligatoridae, as the basal clade, diverged from Crocodylidae — Gavialidae clade approximately 87 mya (million years ago) (Oaks, 2011). The clade Crocodylidae is the youngest member of the order, for which indubitable fossil evidence is first reported from the late Miocene, and it is the most diverse and widespread clade (Oaks, 2011; Rio & Mannion, 2021).

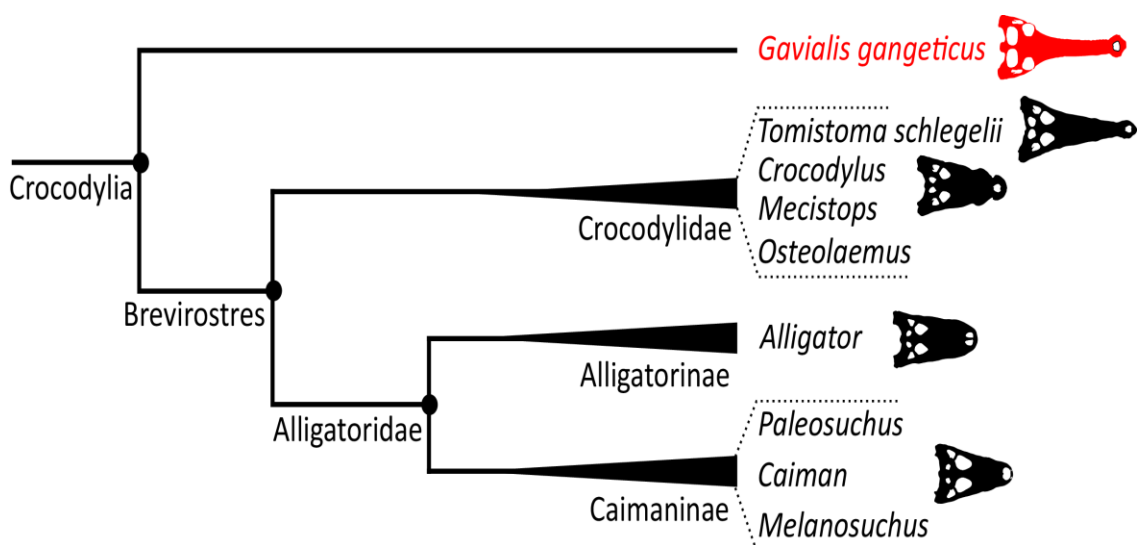


Figure 1.3: Crocodylia cladogram based on morphological dataset illustrating the extant crocodylian interrelationships (Rio & Mannion, 2021).

Table 1.1: List of recognized crocodylian species and their IUCN status (IUCN-CSG, Oaks 2011). LC- Least concern, CR- Critically Endangered, VU- Vulnerable.

Family	Common Name	Species Name	IUCN Status
Alligatoridae	American Alligator	<i>Alligator mississippiensis</i>	LC
	Chinese Alligator	<i>Alligator sinensis</i>	CR
	Spectacled Caiman	<i>Caiman crocodilus</i>	LC
	Broad-snouted Caiman	<i>Caiman latirostris</i>	LC
	Yacare Caiman	<i>Caiman yacare</i>	LC
	Black Caiman	<i>Melanosuchus niger</i>	LR
	Cuvier's Dwarf Caiman	<i>Palaeosuchus palpebrosus</i>	LC
	Schneider's Smooth-fronted Caiman	<i>Palaeosuchus trigonatus</i>	LC
Crocodylidae	American Crocodile	<i>Crocodylus acutus</i>	VU
	African Slender-snouted Crocodile	<i>Mecistops cataphractus</i>	CR
	Orinoco Crocodile	<i>Crocodylus intermedius</i>	CR
	Australian Freshwater Crocodile	<i>Crocodylus johnstoni</i>	LC
	Philippine Crocodile	<i>Crocodylus mindorensis</i>	CR
	Morelet's Crocodile	<i>Crocodylus moreletii</i>	LC
	Nile Crocodile	<i>Crocodylus niloticus</i>	LC
	New Guinea Freshwater Crocodile	<i>Crocodylus novaeguineae</i>	LC
	Mugger Crocodile	<i>Crocodylus palustris</i>	VU
	Saltwater Crocodile	<i>Crocodylus porosus</i>	LC

	Cuban Crocodile	<i>Crocodylus rhombifer</i>	CR
	Siamese Crocodile	<i>Crocodylus siamensis</i>	CR
	Dwarf Crocodile	<i>Osteolaemus tetraspis</i>	VU
Gavialidae	Gharial	<i>Gavialis gangeticus</i>	CR
	Tomistoma	<i>Tomistoma schlegelii</i>	VU

Source: (IUCN, <http://www.iucnscg.org/pages/Crocodylian-Species.html>)

Recent studies based on molecular datasets show a well-supported tree and resolve the long-debated inter-specific relationship of *Tomistoma schlegelii* in crocodylian systematic (Oaks, 2011; Rio & Mannion, 2021). Studies have unambiguously placed *T. schlegelii* as a sister to the *G. gangeticus* within the subfamily Gavialinae in the family Crocodylidae (Janke et al., 2005; Oaks, 2011). Gavialidae is now represented by two species, i.e., *T. schlegelii* and *G. gangeticus* (Figure 1.2).

The proposed reshuffling and revision to place all extant crocodilian species in the two families Alligatoridae and Crocodylidae instead of three traditionally recognized families Alligatoridae and Crocodylidae and Gavialidae within the order Crocodylia is yet to be recognized by crocodile biologists, members of Crocodile Specialist Group (CSG) and IUCN (International Union for Conservation of Nature).

1.4 GEOGRAPHICAL DISTRIBUTION

The crocodylians primarily inhabit the aquatic ecosystem in the tropical and subtropical regions of Asia, Australia, Africa, and the New World, including two

members of the family Alligatoridae viz., American alligator (*Alligator mississippiensis*) and the Chinese alligator (*Alligator sinensis*) inhabiting temperate regions (Grigg & Kirshner, 2015; Somaweera et al., 2020). The gharial (*Gavialis gangeticus*), the mugger or marsh crocodile (*Crocodylus palustris*), and the saltwater crocodile (*Crocodylus porosus*) are three species of crocodylians found in India (Bustard, 1975; Grigg & Kirshner, 2015; Hussain, 1999). The mugger and saltwater crocodile are members of the Crocodylidae family, and the gharial is a member of the Gavialidae family.

The mugger is found throughout the Indian subcontinent and parts of eastern Iran, while the saltwater crocodile is distributed throughout the coast of Sri Lanka, parts of the east coast of India, Myanmar, Thailand, Cambodia, Malaysia, Papua New Guinea, the northern coast of Australia (Da Silva & Lenin, 2010; Webb et al., 2021). On the other hand, the gharial has the narrowest and most isolated distribution, occurring in only a few river systems in the Indian subcontinent (Hussain, 2009) (Figure 1.4).

1.5 THREATS

The crocodylian diversity and population have been fluctuating since the Cretaceous in response to climatic variations (Brochu, 2003; Green et al., 2014; Groh et al., 2020). The demographic reconstruction of effective population size based on the whole genome sequence revealed that gharial experienced a steady decline since their origin. However, a step decline in effective population size was observed between ~5000-10000 kya (thousand years ago) (Green et al., 2014). The demographic

reconstruction of saltwater crocodiles also showed a similar pattern of step decrease in effective population size $\sim 5\text{--}10$ kya following a period of population expansion between $\sim 0.5\text{--}1$ mya. The cause of the decline was majorly attributed to variations in climatic conditions.

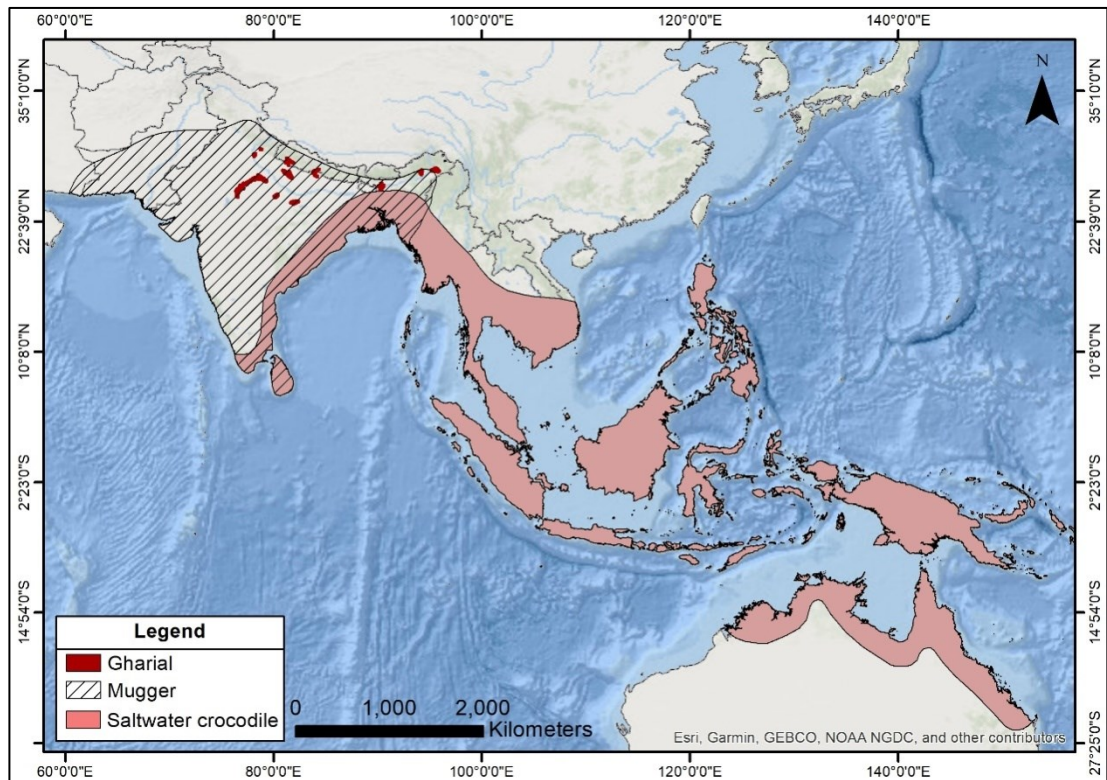


Figure 1.4: Map showing the extant distribution of gharial (*Gavialis gangeticus*), mugger (*Crocodylus palustris*), and saltwater crocodile (*Crocodylus porosus*).

The recent and major decline in the crocodylian population was observed since the 1800s mainly due to intensive hunting (Rossi et al., 2020) before the enactment of national and international laws prohibiting the trade of crocodile parts (Hussain, 1999, 2009; Singh, 1985). At present anthropogenic drivers rather than any natural drivers are the major threats to the survival of crocodylians in India and globally. The key threats to the survival of crocodylians in the wild are increased reliance on river

resources, rapid development activities including building dams, barrages, irrigation canals, artificial embankments, sand mining, riparian agriculture, pollution, and mortality from fishing activities (Katdare et al., 2011).

1.6 STUDY SPECIES

1.6.1 Gharial (*Gavialis gangeticus*)

The gharial is a critically endangered crocodylian, endemic to the Indian subcontinent, characterised by its unique narrow-long cylindrical snout with a bulbous growth at the tip of the snout, which resembles earthen pot ‘*ghara*’ in mature adults (Grigg & Kirshner, 2015)(Plate 1.1). They are the only crocodylian species with sexual dimorphism where males and females can be distinguished based on morphological character (Grigg & Kirshner, 2015; Singh, 1978).



Plate 1.1: An adult male gharial (*Gavialis gangeticus*) basking on the shallow water near river bank.

Until the early twentieth century, the gharial was abundant in the Indus, Ganga, Mahanadi, Brahmaputra, Kaladan, and Irrawaddy Rivers systems in Pakistan, India, Nepal, Bangladesh, Bhutan, and Myanmar, respectively (Hussain, 1991). Over the years, the gharial population has declined by more than 80% and experienced substantial range contraction due to habitat loss, hunting, poaching, and mortalities in passive fishing (Katdare et al., 2011; Nair et al., 2012; Whitaker & Basu, 1982). The extant gharial population is restricted to a few river systems in India, Nepal, and Bangladesh, and it is extinct in Myanmar, Bhutan, and Pakistan (Lang et al., 2019).

The gharial population in Chambal River within the National Chambal Sanctuary and Girwa River within the Katarniaghat Wildlife Sanctuary are the two largest gharial population that holds more than 80% of the global adult gharial population (Lang et al., 2019). These two populations were among a few breeding populations that existed during a range-wide population survey conducted during the early-1970s and served as the major source population for the ‘head-start’ assisted translocation program (Basu, 1981; Singh, 1978).

Since the start of the crocodile conservation program in 1975, population monitoring has been conducted to evaluate the effectiveness of the conservation measures. The first crocodile population census in the Chambal River was conducted in 1979 and reported 107 gharials of all size classes and sexes (Hussain, 1999; Singh, 1985). In the recent population census, a total of 1857 gharials of different ages and sexes were sighted (WII-Chambal, 2020). The Girwa River population has a small population of approximately 34 individuals of different ages and sexes in the early-1980s (Whitaker & Basu, 1982), and as per recent records, 72 individuals of all ages

and sexes continue to exist in the ~20 km stretches of the Girwa River falling within the Katarniaghat Wildlife Sanctuary (Vashistha et al., 2021).

1.6.2 Mugger (*Crocodylus palustris*)

The mugger (*Crocodylus palustris*) is a member of the family Crocodylidae and a primary inhabitant of inland water systems, including freshwater lakes, marshes, and rivers (Plate 1.2). The mugger is highly adaptable and resilient. They can adapt to a range of aquatic habitats and are known to occur in natural systems such as rivers and wetlands and in modified habitats such as reservoirs, irrigation canals, drainages, and artificial ponds (Grigg & Kirshner, 2015). Although they are freshwater species, they have also been reported from coastal saltwater lagoons and estuaries (Whitaker, 1987; Whitaker & Andrews, 2003). They also easily adapt to new environments and have been found in reservoirs, irrigation canals, and other human-made freshwater bodies (Da Silva & Lenin, 2010).



Plate 1.2: Adult mugger (*Crocodylus palustris*) basking on the flat sand bar.

The mugger is among the most widely distributed crocodylian in Asia, which has also suffered a massive population decline, with some populations experiencing extirpations during the late-1960s (Bustard, 1975; Jacobson, 1999). The mugger population, like the gharial, has recovered as a result of cumulative conservation measures begun in the mid-1970s. In the Chambal River, the mugger population was reported to be 33 individuals of all size classes and sexes in 1985 (Singh, 1985). The current population of the mugger crocodile in the Chambal River is 674 (WII-Chambal, 2020), and Girwa is 42 of all size classes and sexes (Choudhary et al., 2017).

1.7 CROCODYLIAN CONSERVATION IN INDIA

The crocodylian population in India had plummeted by the early 1960s owing to hunting for hides and habitat loss, prompting authorities to list the species as Schedule I under the Indian Wild Life (Protection) Act, 1972, which prohibits killing and trade of the listed species (Bustard, 1975). The other reason for the decline is the high mortality rate experienced in small classes. The crocodylian hatchlings are reported to suffer massive mortality of upto 97% in the wild, almost entirely attributed to predation and the wipe-off during the floods (Hussain 1999). Hence, an active management effort was needed to revive the declining crocodylian population in India. Subsequently, a conservation program was initiated in the mid-1970s to restore the species through the ‘head-start’ assisted conservation translocation (Bustard, 1975, 1976). The ‘head-start’ program involves the collection of wild-laid eggs soon after laying or at the end of incubation and relocating them in an artificial hatchery for hatching. The hatchlings produced were then reared in captivity, and individuals were released back into suitable habitats within indigenous range after attaining a length of

1.2 m (Bustard, 1975; Hussain, 1999; Singh, 1978; Whitaker & Basu, 1982). Within a decade of the initiation of the conservation program, 16 rehabilitation and rearing centers were established across India. Additionally, conservation breeding was initiated in several zoos, such as the Nandankanan Biological Park and Madras Crocodile Bank (Singh, 1982). The gharial 'head-start' was mostly concentrated in the northern and central Indian rivers, whereas mugger was released in Gujarat and Tamil Nadu with scattered records in northern Indian states (Singh, 1985; Whitaker, 1987). The gharial and mugger populations in India have largely been recovered through a conservation translocation program and thriving well where there is suitable habitat and which has been supplemented continuously. The head-start strategy has been practiced since the mid-1970s, and juvenile gharial reared in rehabilitation has been translocated to suitable habitats. So far, over 5000 juvenile gharials have been released in more than 12 Indian rivers. The current global gharial population is estimated to be below 900 mature individuals (Lang et al., 2019), while the global mugger population is estimated to be above 5700 mature individuals (Choudhury & de Silva, 2013).

1.8 ROLE OF CONSERVATION GENETICS IN SPECIES RECOVERY

The Convention on Biological Diversity has recognised genetic diversity as a fundamental component of biodiversity and ecological and species diversity (Hoban et al., 2021). The genetic contribution to species persistence cannot be concealed; numerous theoretical and empirical studies have demonstrated that genetic diversity, whether adaptive or neutral, affects the rate of adaptation in populations, and species or populations with limited genetic variation are extremely susceptible to extinction (Frankham, 2005; Spielman et al., 2004). The genetic diversity measured at neutral

loci is regarded as a reasonable approximation of species' adaptive potential; hence, assessment of neutral genetic diversity provides insights into populations' adaptive and evolutionary potential and/or species adaptability and long-term persistence.

Genetic analysis can be a valuable tool in wildlife conservation efforts, providing information on the biology, behavior, and distribution of species and population genetics and the relationships between different populations. This information can be used to develop and implement conservation strategies, such as monitoring populations, managing habitats, and controlling the spread of diseases. Additionally, genetic analysis can help detect and understand the impacts of human activities, such as habitat fragmentation, the introduction of non-native species, and climate change, on wildlife populations. Genetic analysis can also help prioritize conservation efforts and guide decision-making in wildlife management by providing insights into the evolutionary history and diversity of species.

The genetic variation in species, population, and individual is measured through several polymorphic sites, alleles, and heterozygosity (Allendorf et al., 2012; Charlesworth, 2003). Genetic diversity in a population is dynamic, where small and isolated populations are more vulnerable and tend to suffer a higher degree of loss of genetic variation as a result of balancing between stochastic events (Frankham et al., 2014; Reed & Frankham, 2003). The loss of genetic variation in the ecological time scale is unidirectional (Lacy, 1987). The intensity of such loss increases, and the population experiences a greater impact of genetic drift in the absence of balancing forces such as gene-flow, which makes species more vulnerable to the harmful effects of reduced genetic diversity (Furlan et al., 2012). On the other hand, a population that

has suffered a bottleneck is often characterised by reduced genetic diversity because of the loss of rare alleles or gene variants (Frankham et al., 1999; Lacy, 1997). Each of these factors alone or in combination results in loss of genetic diversity, and species or populations showing any of these factors are likely to suffer negative consequences of low genetic diversity such as ‘inbreeding’ (Reed & Frankham, 2003). In a nutshell, inbreeding in the wild has multiple short- and long-term effects. In the short term, inbreeding affects diversity and increases the frequency of homozygosity in the population. In the long term, it reduces effective population size, reduces the effective frequency of recombination, and ultimately reduces the evolutionary potential (Charlesworth, 2003). The effect of inbreeding is reported to increase in species with complex life-history traits, such as male territoriality, dominance, and philopatry (Ellegren & Galtier, 2016; Muniz et al., 2011).

In the last few centuries, anthropogenic factors, rather than any natural forces, have emerged as the primary threat to the survival of many plant and animal species (Bongaarts, 2019). They have resulted in the extinction of several species, while many others have reduced in population sizes and range, making them vulnerable to extinction. The threats to wildlife populations, such as habitat loss, degradation, fragmentation, over-exploitation, the introduction of non-native species, and climate change, can interact and compound each other, resulting in a synergistic negative impact on populations. In such a scenario, the genetic diversity of a species can become a crucial factor in its ability to adapt and respond to these threats. A high level of genetic diversity allows for greater evolutionary flexibility, allowing species to adapt to changing environmental conditions and resist diseases. On the other hand, a lack of

genetic diversity can limit a species' ability to respond to environmental changes, making it more vulnerable to extinction. Therefore, maintaining genetic diversity is a critical aspect of wildlife conservation efforts, as it can help ensure the long-term survival and persistence of populations and species. This has sparked a global sense of emergency and prompted taking conservation decisions considering genetics as an important component to protect these species from extinction. In the last five decades, there has been a lot of interest in wildlife conservation and species recovery, which has led to an exponential increase in conservation strategies such as '*Conservation translocation*' for reversing the species decline. However, the low success of these strategies remains a challenge in the field of conservation (Berger-Tal et al., 2020).

Not every translocation program becomes a success, and effective translocation programs essentially depend on prior knowledge of the species' ecology and biology, habitat requirement, availability of suitable habitat, climatic conditions, behavior, life-history trait, demographic, genetics, and adequate post-release monitoring (IUCN/SSC, 2013; Schwartz, 2005; White et al., 2018). Recent studies have analysed the low success rate of translocation programs worldwide and highlighted the role of demographic and genetics monitoring of translocated populations in improving program success (Berger-Tal et al., 2020; Weeks et al., 2011). Monitoring the translocated population provides opportunities to measure the program's efficacy and implement suitable methods to enhance the success of these efforts (IUCN/SSC, 2013). Nonetheless, many of these programs either lack any monitoring mechanism or rely solely on demographic monitoring to evaluate the effectiveness and success of the program without considering genetics. Thus, many programs remain devoid of genetic

information, which is crucial in planning conservation policies and designing translocation programs (DeMay et al., 2017; Laikre et al., 2010; Pacioni et al., 2019; Schwartz, 2005).

1.9 SIGNIFICANCE OF THE STUDY

Crocodylians are among the most studied species globally, literature can be traced back to the 1800s. Crocodylian research has increased considerably since the late-1950s, and crocodylian conservation has garnered much attention (Singh, 1978). Tremendous efforts have been spent on protecting these species in the wild, and the present gharial and mugger population in India have primarily recovered due to these conservation efforts initiated under ‘Project Crocodile’ in the mid-1970s (Bustard, 1975; Whitaker, 1987). Since the inception of the conservation program, several studies have been undertaken; in India, early crocodylian studies were mainly focused on aspects of population dynamics (Hussain, 1999, 2009; Singh, 1985). Later with the growing interest and inflow of funds in crocodylian research, the breadth of the studies undertaken was broadened to incorporate aspects of systematic, biology, and ecology of the species (Katdare et al., 2011; Vashistha et al., 2021)

Despite the recognized importance of genetics in the conservation and management of small, isolated, and recovered populations, no information is available on the genetic status of these sympatric crocodylian species (Allendorf et al., 2012). The lack of genetic information on these species has hindered, if not precluded, effective conservation planning. It is also essential to determine the loss of genetic

variation over space and time through genetic monitoring to develop effective conservation planning for the species (Weeks et al., 2011).

The study of the genetic status of gharial and mugger is particularly important due to its population decline and subsequent demographic recovery through 'head-start' assisted conservation translocation and associated conservation efforts. The gharial population in the Chambal and Girwa river account for up to >80% of the adult population and up to 90% of nests in the wild (Lang et al., 2019). The mugger population in the Chambal River is also the single largest population in any river. Hence, genetic assessment of these populations is particularly crucial, as they continue to act as the source population for the ongoing gharial conservation translocation effort. Currently, there is no study on the assessment of the genetic diversity of these species and other aspects of population genetics such as population genetic structure, gene-flow, and mating system that are crucial to understanding and predicting long-term survival and necessary for conservation management. However, studies on developing novel microsatellite markers and cross-species amplification of microsatellite markers (Aggarwal et al., 2014; Jogayya et al., 2013) have been attempted earlier.

In the recent past, the gharial and mugger throughout their distribution range are understood to have suffered from population decline (Hussain, 1999, 2009; Lang et al., 2019). Inbreeding due to small population sizes is a serious threat to populations; hence, it is essential to understand the loss of genetic diversity in the current population.

1.10 LITERATURE REVIEW

1.10.1 CROCODILE CONSERVATION IN THE PAST

The gharial and mugger populations experienced severe declines and substantial range contraction between late-19th and mid-20th centuries (Bustard, 1975, 1976). The cause of the decline was majorly attributed to hunting, habitat destruction, poaching, and mortalities in passive fishing (Bustard, 1975; Hussain, 2009; Lang et al., 2019). By the late-1970s, the largest known population of gharial was the one in the Chambal River, with 107 individuals and 34 individuals from Girwa River of all sizes, classes, and sexes (Whitaker & Daniel, 1980).

The gharial is the most threatened species among all other crocodylian species occurring in the Indian sub-continent and has the narrowest distribution, with few isolated populations occurring in India, Nepal, and Bangladesh (Lang et al., 2019). The species is believed to be extinct in Bhutan (last recorded in 1980), Myanmar (last recorded in 1976), and possibly a small number of individuals occurring in Pakistan and Bangladesh (Ahmad, 1990; Bustard, 1980; Chaudhry, 1993; Cox & Rahman, 1994; Stevenson & Whitaker, 2010; Webb & Smith, 1987). While the mugger has a much wider distribution, they are found in the southern-eastern part of Iran, most of the southern part of central Pakistan, the central and southern part of northern India, southern Nepal, and throughout Sri Lanka (Da Silva & Lenin, 2010). Mugger is also considered to be extinct from Bhutan (Whitaker & Andrews, 2003) and extinct in the wild in Bangladesh (Cox & Rahman, 1994) and Rahman 1994) and last was reported in 1987 in Myanmar (Da Silva & Lenin, 2010).

Subsequent to the range-wide catastrophic decline, all three species of crocodylians occurring in India were listed in Schedule I of the Indian Wild Life (Protection) Act, 1972, to provide enhanced protection (Bustard, 1975; Whitaker, 1987). Later during the decade, a crocodile conservation program was initiated with the following objectives (Bustard, 1999; Singh, 1999):

- a) To protect the remaining population of crocodilians in their natural habitat by creating sanctuaries.
- b) To rebuild natural population quickly through 'head-start' or 'grow and release' or 'rear and release' assisted conservation translocation.
- c) To promote captive breeding.
- d) To take up research and build up a trained workforce to improve management.

Although the conservation effort was begun for all three species of crocodiles found in India, the gharial has received the most attention. The program was first launched in the state of Odisha, which has been home to all three crocodylian species (Bustard, 1999). Later, the program was extended to other parts where suitable habitats and adult breeding populations existed. Over the decade (1975-1985), multiple crocodile rehabilitation centers and wildlife sanctuaries were established by the Government of India to prevent any further population decline in the wild (Singh, 1985). Few of them were established dedicated for the recovery of gharial, viz. (i) Gharial Rehabilitation Centre, Tikarpada; (ii) Gharial Rehabilitation Centre,

Katerniaghat; (iii) Gharial Rehabilitation Centre, Kukrail; and (iv) Deori Gharial Rearing Centre, Morena targeting the Mahanadi, Girwa and Chambal populations.

Gharial eggs were obtained from Chambal and Girwa rivers in 1975 and reared in the Kukrail Centre. Other facilities began to operate in later years (Basu, 1981; Singh, 1978). The Kukrail and Deori center became the key facilities for gharial conservation due to their proximity to the major gharial population (Plate 1.3)

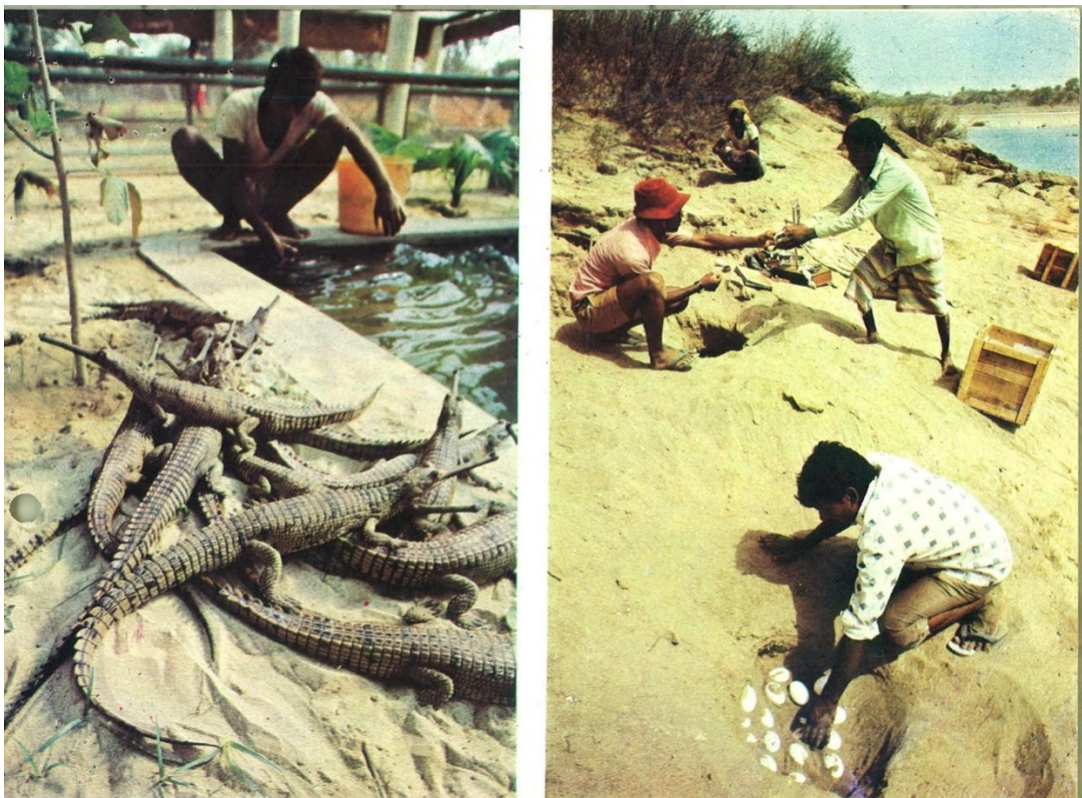


Plate 1.3: Right: Collection of wild laid eggs for the ‘head-start approach-based translocation program from the Chambal River. Left: Gharial yearlings and juveniles produced in the Kukrail center from wild-collected eggs (Source: Basu, 1981).

The hatchlings produced in Kukrail and Deori were also translocated to several other rivers. Son, Ken, and Ramganga, some of which have started breeding (Stevenson & Whitaker, 2010). Eggs were also collected from the Narayani River in

Nepal, and hatchlings were released in the Girwa river. Additionally, conservation breeding was initiated in several zoos, such as the Nandankanan Biological Park and Madras Crocodile Bank, which contributed stocks for translocation (Whitaker & Basu, 1982). A male gharial from the Frankfurt Zoo, Germany, was obtained, contributing to developing stocks in Odisha for translocation (Whitaker, 2007). All translocation sites are now protected as wildlife sanctuaries under the Indian Wild Life (Protection) Act, 1972. In the last four decades, over 5000 gharial individuals have been translocated in more than 12 rivers in India, with more than 3500 juvenile gharial in the Chambal and Girwa Rivers (Whitaker, 2007).

The gharial population in the Chambal River, which was once 107 individuals at the start of the restocking program, has now grown to approximately 1675 individuals of varying ages and sexes (Hussain, 1999; Lang et al., 2018). The Chambal River, within the National Chambal Sanctuary that spans over 425 km, has become a significant hub for the gharial population since the start of the crocodile conservation program in the mid-1970s. The Girwa River, which initially had only 34 individuals of varying ages and sexes (Whitaker & Basu, 1982), now boasts a population of 43 adult individuals restricted to a 20 km stretch within the Katarniaghat Wildlife Sanctuary. Currently, these two populations make up more than 80% of the surviving wild gharial populations (Lang et al., 2019).

1.10.2. PRESERVATION OF GENETIC DIVERSITY

The recognition of genetic diversity as one of the components of biological diversity along with species and ecosystems by the Convention on Biological Diversity (Hoban, Campbell, et al., 2021). It has profoundly changed how genetic diversity was

perceived before. Nations and organisations are now determined and striving to preserve all the component biodiversity, have recommended the protection of genetic diversity, and suggested legislation to maintain the level of genetic diversity. Multiple guidelines and frameworks have been developed to consider monitoring as an essential component for the preservation of genetic diversity effectively (Hoban, Bruford, et al., 2021).

Genetic diversity is essential as these estimates correlate with species' adaptive potential and reflect the population's magnitude of inbreeding and genetic drift (Allendorf et al., 2012; Hoban, Gaggiotti, et al., 2013). Loss of genetic diversity has several potential adverse consequences that impact the resilience and adaptability of individuals, populations, or species (Garner et al., 2005). The consequences are worrisome, and the magnitude is higher in isolated and small populations due to loss of genetic variation and genetic drift (Reed & Frankham, 2003). Mitochondrial DNA (mtDNA) and nuclear DNA are powerful and effective neutral genetic markers for studies of genetic variation, population genetic structure, gene-flow, and parentage analysis (Holderegger et al., 2006; Sunnucks, 2000; Wan et al., 2004).

Various life-history traits have been reported to safeguard from negative consequences and help maintain the genetic diversity in species (Allendorf et al., 2012). For instance, species showing the incidence of multiple paternity have been reported to have high effective population size, increased genetic variation, and evolutionary potential (Chesser & Baker, 1996; Sugg & Chesser, 1994), thus acting as potential mechanisms to maintain the overall genetic diversity in populations. The incidence of multiple paternity is believed to have increased with population size

decline, especially in populations that have recently witnessed a genetic bottleneck (Karl, 2008; Uller & Olsson, 2008; Young & Brown, 1999). However, it's important to note that multiple paternity is not solely the result of population size decline and can also occur in larger populations for a variety of other reasons, such as differences in the mating behaviors of males, sperm competition, and changes in the social structure of a population. Microsatellites are effective and robust, with the ability to identify individuals and detect population genetic structure and analysis of mating systems (Holderegger et al., 2006; Sunnucks, 2000; Wan et al., 2004).

Hence, a combination of demographic, genetic, and ecological studies are necessary to evaluate the translocation program's effectiveness and population status.

1.11 OBJECTIVES AND RESEARCH QUESTIONS

The overall aim of the study was to assess the genetic diversity, population genetic structure in the two sympatric crocodylian species in Chambal and Girwa river, and the incidence of multiple paternity as a mechanism to counter balance the loss of genetic diversity. The genetic information of these species is essential for developing a comprehensive species conservation and management plan and guiding the ongoing conservation translocation program. The objective and research question of the study are as follows:

- a. To examine the genetic variation among the gharial and mugger populations using mitochondrial and nuclear DNA markers.
 - What is the genetic status of the largest managed gharial population?
 - Do the two populations differ in terms of genetic diversity?

- b. To assess the gene-flow pattern among the gharial and mugger populations within the National Chambal Sanctuary and adjacent habitat (Katarniaghat Wildlife Sanctuary).
- Do the Chambal and Girwa river populations genetically connected?
Do they have population-specific genetic signatures?
 - If so, what is the level of differentiation?
 - Do the gharial and mugger also support the ‘crocodylan-philopatry’ theory?
- c. To investigate the incidence of multiple paternity in gharial and mugger in National Chambal Sanctuary.
- Do they adopt multiple paternity as a behavioral mechanism to counter low genetic diversity, low effective population size, and inbreeding?

1.12 HYPOTHESIS

- (a) The genetic diversity in the Chambal River population would be higher than the Girwa River population.
- (b) Following crocodile life history traits: philopatry- it is likely to observe a structured genetic population and a higher level of differentiation with a low level of gene-flow between sites.
- (c) The extant crocodylians are characterized by demographic decline and display a complex hierarchical social structure and philopatry. All these

factors are evident in reducing the genetic diversity in species. Hence, both species are likely to show an incidence of multiple paternity.

1.13 STUDY AREA

In India, by the early 1970s, the gharial was restricted to a few isolated locations in the Ganga, Mahanadi, and Brahmaputra River systems. The Chambal River within the National Chambal Sanctuary has been a stronghold and, along with Girwa River populations, were among a few breeding populations that existed during a range-wide population survey conducted during the early-1970s and served as the major source population for the ‘head-start’ supported translocation program initiated in the mid-1970s (Bustard, 1975, 1980).

1.13.1 NATIONAL CHAMBAL SANCTUARY (IN CHAMBAL RIVER)

The Chambal River and its surrounding areas are of significant ecological importance, and the establishment of the National Chambal Sanctuary was a substantial step in conserving the aquatic fauna found in the river. The sanctuary provides a protected habitat for the critically endangered gharial, mugger, and Gangetic dolphin, as well as other important species of freshwater turtles. The Chambal River originates in the Vindhya Hill Range in central India (Figure 1.5). It flows north-easterly through Madhya Pradesh, Rajasthan, and Uttar Pradesh before it meets the Yamuna River. Since 1979, a ~600 km stretch of the Chambal River between Kota barrage in Rajasthan and the Chambal –Yamuna confluence in Uttar Pradesh and a ~60km stretch of Parbati river in Madhya Pradesh (south bank) between Badodiya Bindi and Pali has been protected as National Chambal Sanctuary for the

conservation and management of aquatic fauna such as gharial (*Gavialis gangeticus*), mugger (*Crocodylus palustris*), dolphin (*Platanista gangeticus*), and freshwater turtles. The Sanctuary is also the longest riverine protected area in India. The boundary of the Sanctuary extends for one km from mid-river on both the banks in Madhya Pradesh and Rajasthan and the south bank in Uttar Pradesh. The northern bank boundary of the Sanctuary in Uttar Pradesh is marked by a road running parallel to the Chambal River until it meets the Yamuna River and after that, the south bank of the Yamuna River until the Chambal—Yamuna confluence.

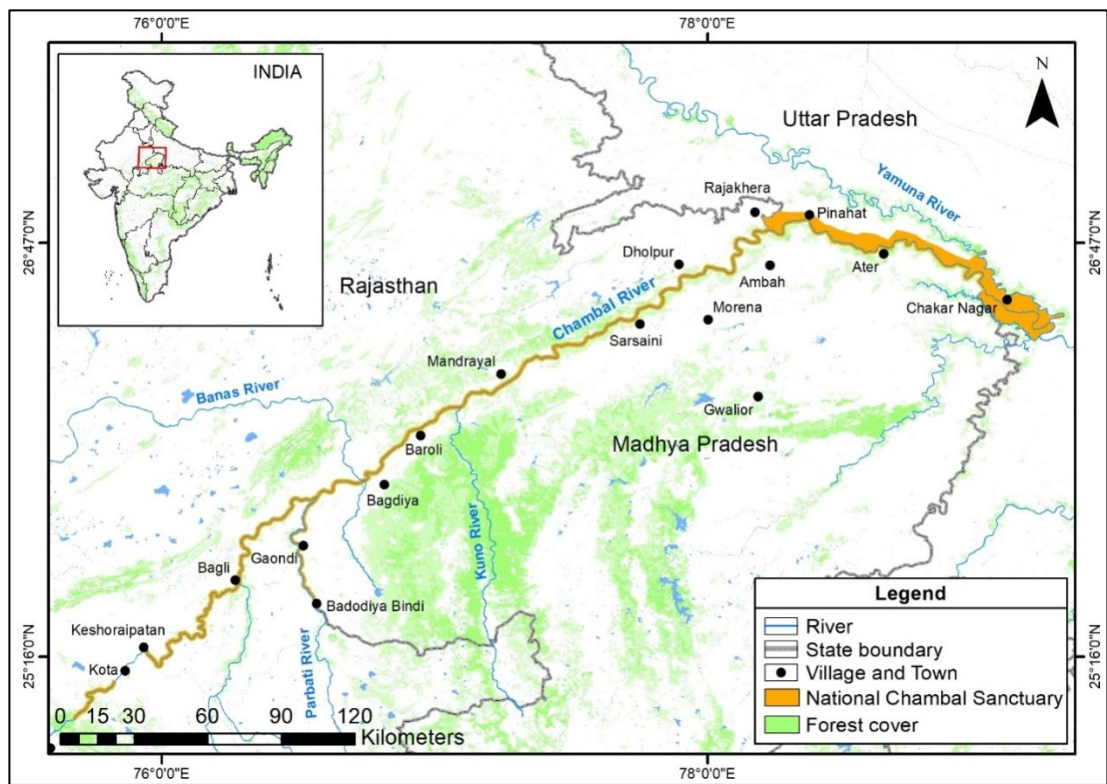


Figure 1.5: Map showing National Chambal Sanctuary (orange color) within Chambal River. A ~600km of the Chambal River is protected to conserve the aquatic fauna.

The Sanctuary administrative limits are under the authority of the States of Rajasthan, Madhya Pradesh, and Uttar Pradesh and are administered by the relevant State Forest Departments (Hussain, 1991).

1.13.2 KATERNIAGHAT WILDLIFE SANCTUARY (IN GIRWA RIVER)

Katerniaghat Wildlife Sanctuary (KWLS) is a protected area located close to the Indo-Nepal border in the Upper Gangetic plain and represents part of the Terai ecosystem in the foothills of Himalaya (Figure 1.6).

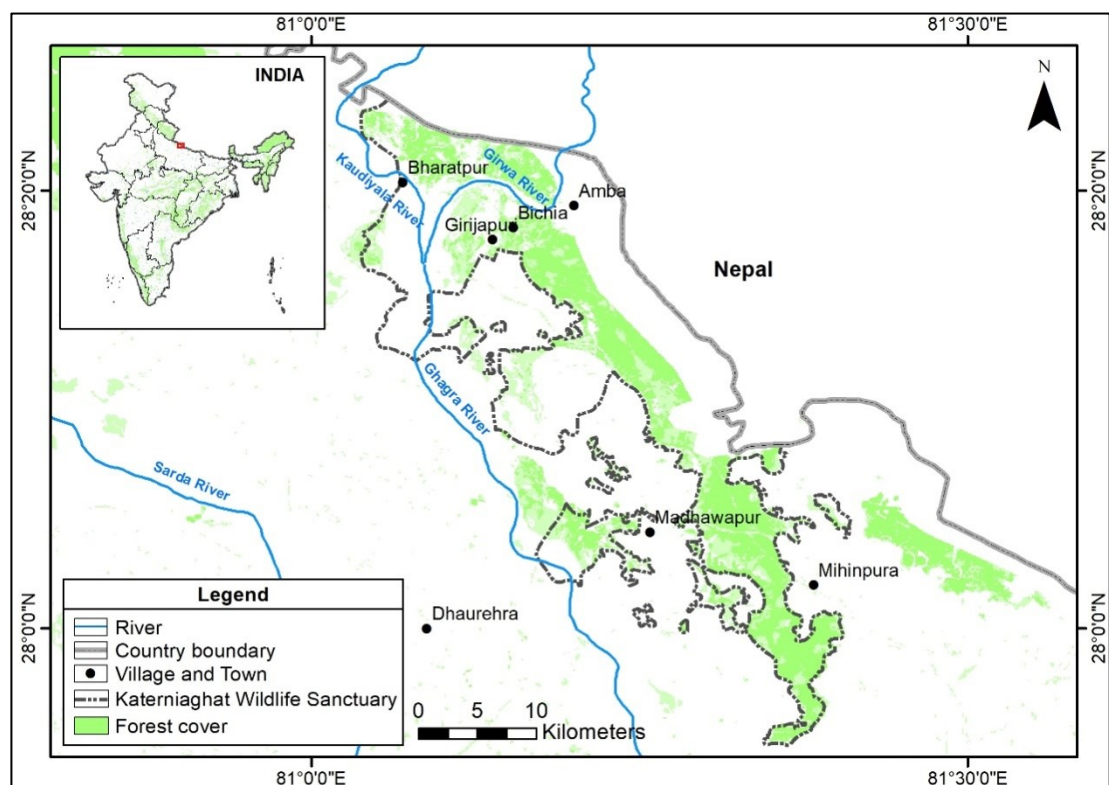


Figure 1.6: Map showing Katerniaghat Wildlife Sanctuary (dashed line). A 20km stretch of Girwa River within the Katerniaghat Wildlife Sanctuary is prime habitat for gharial.

The Sanctuary was established in 1975 (Choudhary et al., 2017) and span over 400 sq km. Two decades after its establishment, the Sanctuary was amalgamated with Dudhwa National Park and Kishanpur Wildlife Sanctuary to form the Dudhwa tiger

reserve. Besides providing suitable habitat for threatened mammals like River dolphin (*Platanista gangetica*), tiger (*Panthera tigris*), elephant (*Elephas maximus*), rhino (*Rhinoceros unicornis*), Barasingha (*Rucervus duvaucelii*), a ~20km stretch of the Girwa River that flows through the KWLS, between Indo-Nepal border to the Girwa — Kaudiyala confluence is a prime habitat for gharial and mugger. The stretch is one of the few gharials breeding sites in India (Vashistha et al., 2021).

1.14 ORGANISATION OF THE THESIS

The thesis is organized into five chapters. Chapter 1 provides a brief overview of crocodylians, including the origin and evolution of crocodylians, geographical distribution, conservation overview and general information of the study species, the role of genetics in species recovery, significance of the study, review of literature, aim, and objectives of the study and the study area. Chapter 2 deals with the genetic assessment of the largest sympatric freshwater crocodylian population in the National Chambal Sanctuary (in Chambal River) using mitochondrial and nuclear markers. Chapter 3 discusses the factors that may influence the level of genetic diversity and connectivity with the Katarniaghat population (in the Girwa River). Chapter 4 attempts were made to unravel the complex mating system in the two threatened freshwater crocodylian species. Chapter 5 summarizes the findings of my research and conservation implication.

CHAPTER 2

GENETIC VARIATION USING NUCLEAR MICROSATELLITE AND MITOCHONDRIAL DNA REGION

2.1 SUMMARY

The crocodylians throughout its range have experienced a severe population decline and range contraction starting in the early ninetieth century, which intensified in the mid-twentieth due to hunting, habitat loss, poaching, and mortalities in passive fishing. The extant crocodylian populations in India have been mainly recovered through a 'head-start' assisted conservation translocation program initiated in the mid-1970s. The gharial population in the Chambal River has been a stronghold since the initiation of the conservation program. The gharial population in the Chambal River has been the largest single population even before the initiation of the conservation translocation program and has been the primary source for the 'head-start' conservation translocation program. Both species have recovered mainly due to dedicated conservation efforts in the past. Hence, understanding the genetic status of the population suffering substantial decline and recovery is crucial for effective conservation planning. The information on genetic status is also essential for monitoring the recovery of the population and is among the parameters essential for evaluating the effectiveness of ongoing conservation efforts. This study assessed the genetic diversity of two sympatric crocodylian species inhabiting the Chambal River using nuclear microsatellite and mitochondrial markers. Additionally, n=72 complete mitochondrial genome sequences of 23 crocodylian species were obtained from National Center for Biotechnology Information (NCBI) repository to analyse genetic diversity across the extant crocodylian species.

The mitochondrial control region sequences in gharial were invariable with a single haplotype, while in mugger mitochondrial control region sequences exhibited two haplotypes. The complete mitochondrial genome analysis of 23 crocodylian species using 72 sequences does not yield any association with IUCN conservation status or population size and distribution of the species. In comparison, *C. palustris* (n=4) showed higher genetic variation than *G. gangeticus* (n=3). 228 (65%) gharial samples and 81 (66%) mugger samples were genotyped at seven and ten polymorphic loci. The genetic diversity estimates – heterozygosity and allelic richness in the Chambal gharial population- were lower than in captive gharial estimated using the same set of microsatellite loci. Overall, the gharial population revealed a low genetic diversity compared to the mugger population.

2.2 INTRODUCTION

Extinction and speciation are two natural processes that have been critical components in the progression of evolution (Gaston & Fuller, 2007). Extinction refers to the disappearance of species, while speciation refers to the formation of new species through genetic divergence. Extinction events can occur as a result of natural causes, such as changes in climate, disease outbreaks, and competition for resources, or as a result of human activities, such as habitat destruction, the introduction of invasive species, and overexploitation of resources (Ceballos et al., 2020; Ceballos & Ehrlich, 2002). These processes have shaped the diversity of life on Earth and continue to play a crucial role in shaping the future of biodiversity. The extinction process entails progressive reductions in a species' abundance and geographic range (Gaston & Fuller, 2007). These rates fluctuate over time and often reach a magnitude much higher than

the background rates, due to various stressors such as climate change. The current species extinction rates are hundreds to thousands of times higher than the background rates prevailing in the last millions of years (Ceballos et al., 2020). In the last couple of centuries, an increasing number of theoretical and empirical studies implicate human-driven overexploitation as the root of the accelerating extinction rate (Gaggiotti, 2003; Woodruff, 2001). Human-driven overexploitation in small geological time scales has caused demographic decline, range contraction, population isolation, and extinction of numerous species (Ceballos et al., 2020; Frankham, 2005). Species that suffer from any or combination of these factors are likely to be characterized by low genetic diversity and low population resilience and adaptability (Allendorf et al., 2012).

Genetic diversity is a crucial factor for a species's long-term survival and persistence. The magnitude of genetic diversity within a species and or population is closely linked to its adaptive potential, as it provides the genetic variation necessary for the species to evolve and respond to changing environmental conditions (Ellegren & Galtier, 2016; Gaggiotti, 2003; Reed & Frankham, 2003). Additionally, the level of genetic diversity in a population can also indicate the level of inbreeding and genetic drift, which are processes that can reduce genetic diversity and lead to a loss of adaptive potential (Frankham et al., 1999). The level of genetic diversity in a species is often correlated with its geographical distribution and population size. Large, wide-ranging populations tend to have a higher level of genetic diversity than small, isolated populations (Isagi et al., 2020; Kahilainen et al., 2014). This is because larger populations provide a greater pool of genetic diversity and are less likely to experience

the negative effects of genetic drift, which can lead to a reduction in genetic diversity in small populations. On the other hand, small, isolated populations are more susceptible to the effects of genetic drift and inbreeding, which can reduce genetic diversity and increase the frequency of deleterious alleles. Additionally, small populations are often more vulnerable to stochastic environmental events, such as natural disasters, and stochastic demographic events, such as random fluctuations in birth and death rates, which can further reduce genetic diversity (Furlan et al., 2012). Therefore, it is important to consider the size and distribution of populations when planning conservation efforts, as the genetic diversity level can greatly influence species' long-term survival and persistence. In populations with a small number of individuals, genetic rescue or the introduction of individuals from other populations to increase genetic diversity may be necessary to help ensure the species long-term survival. Populations rapidly lose genetic variability due to various factors such as bottleneck and isolation acting through genetic drift and inbreeding, potentially interfering with the resilience and adaptability of individuals, populations, or species (Allendorf et al., 2012; Ellegren & Galtier, 2016; Garner et al., 2005). The magnitude of genetic loss is more significant in a small population with little or no gene flow. Increased homozygosity and increased frequency of recessive deleterious alleles, known as inbreeding, are immediate effects of reduced variability, which in turn decreases species' evolutionary adaptive potential, sometimes leading to the extinction of a species locally or globally (Ellegren & Galtier, 2016).

The preservation of genetic diversity is widely recognized as an essential component of biodiversity, along with species and ecological diversity (Hoban,

Campbell, et al., 2021). The Convention on Biological Diversity (CBD) recognizes that genetic diversity is the foundation of biodiversity and that the maintenance of genetic diversity is essential for the long-term survival and adaptation of species and ecosystems. The CBD also acknowledges that genetic diversity is essential for the continued evolution of life on earth and for maintaining the resilience and adaptability of ecosystems (Hoban, Hauffe, et al., 2013; Rands et al., 2010). Since then, multiple frameworks and guidelines have been developed to prevent the loss of genetic diversity at the individual, population, and species levels.

The post-2020 Global Biodiversity Framework, which was developed as part of the United Nations Decade on Biodiversity, places a strong emphasis on the importance of genetic monitoring in preventing the loss of genetic (Armitage et al., 2020; Erdelen, 2020; Nicholson et al., 2021). The Framework recognizes that genetic diversity is a critical component of biodiversity and that the loss of genetic diversity can have serious impacts on the ability of species and ecosystems to adapt to changing environmental conditions. The Framework calls for developing and implementing genetic monitoring programs to track changes in genetic diversity over time and identify and respond to threats to genetic diversity. This includes efforts to collect and maintain genetic data, as well as the development of methods for genetic analysis and interpretation (Hoban, Bruford, et al., 2021). The Framework also emphasizes the importance of integrating genetic data into conservation decision-making to support the development of effective conservation strategies. This is an important step towards ensuring the long-term survival and persistence of species and ecosystems, and the

maintenance of the genetic diversity that is essential for their adaptation and evolution.(Hoban, Bruford, et al., 2021).

Conservation strategies that consider all facets of species' biology, ecology, and genetics are, hence, prerequisites for the successful management of the species that are vulnerable due to human-driven overexploitation (Bubac et al., 2019). Genetic diversity is a crucial aspect of species conservation and management. The genetic variation within a species can help determine its adaptability, resilience, and ability to survive in the face of environmental changes (Allendorf et al., 2012). Monitoring the genetic status of the gharial and mugger populations in the Chambal River is crucial for determining the success of population recovery of translocated populations and ensuring the long-term survival of these species (Weeks et al., 2011). The genetic information will also help guide the ongoing conservation translocation program and adapt appropriate measures to protect against further loss of genetic diversity in species managed in regulated rivers within Protected Areas.

2.3 METHODOLOGY

2.3.1 SAMPLE COLLECTION

Potential nest sites were identified during the winter surveys (February-March) either based on the presence of suitable nesting habitat (sandbank with adequate slope), aggregation of a large number of gharial individuals, and previous year nesting records. Once a site was identified as a potential nesting site, follow-up monitoring surveys were conducted at regular intervals (7-15 days) after the egg laying (mid-March) till hatching (May-June).

Biological samples such as tissue from dead hatchlings and chorioallantoic membranes from hatched eggshells were collected shortly after hatching (Plate 2.1). The gharial samples were collected from 16 nesting sites and mugger samples from four nesting sites along the Chambal River (Figure 2.1). All the samples were collected during post-hatching surveys in the month of May-June 2017 and May-June 2018. The samples were stored in absolute ethanol at room temperature and later at -20 °C in the laboratory for long-term storage.



Plate 2.1. Plate showing different biological sample types collected for genetic assessment of gharial and mugger.

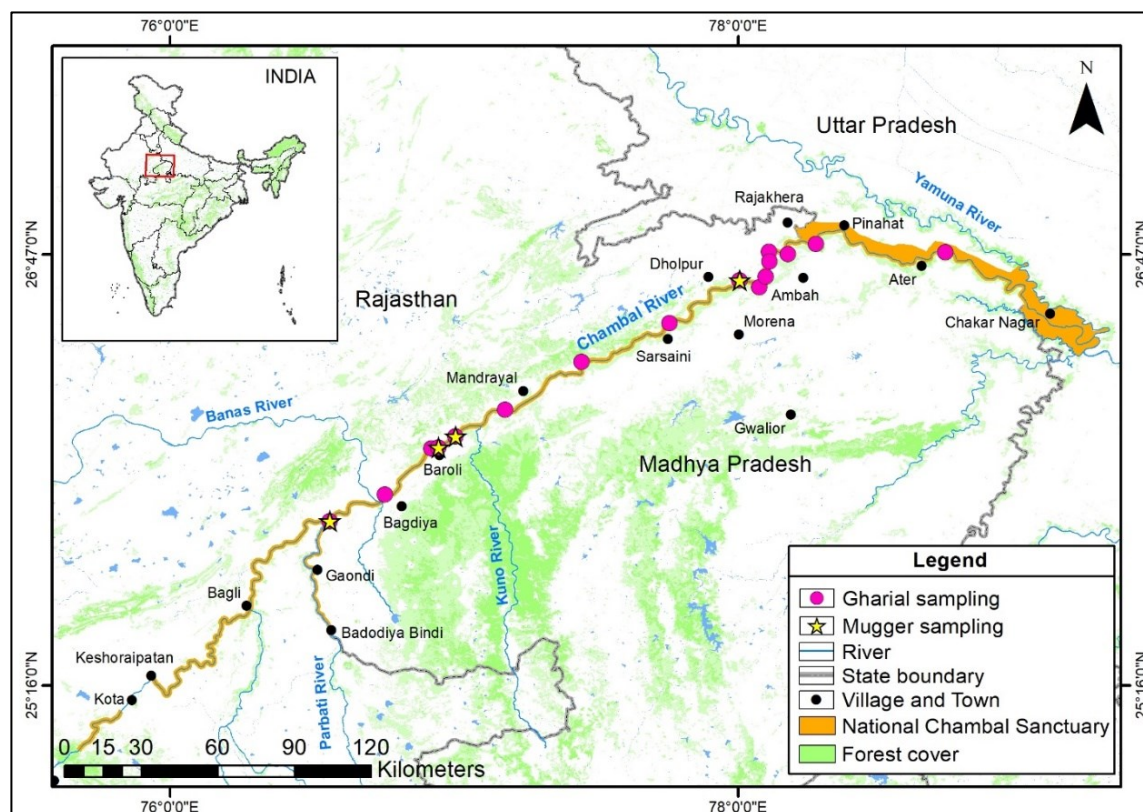


Figure 2.1: Detailed representation of gharial and mugger samples collected for genetic assessment along the Chambal River.

2.3.2 DNA EXTRACTION

Total genomic DNA was extracted from tissue and chorioallantoic membrane using the phenol-chloroform method (Sambrook et al., 1989) (Appendix 1). Cotton swabs were used when the chorioallantoic membrane was unable to separate from eggshells or failed to recover DNA.

2.3.3 SELECTION AND SCREENING OF MARKERS

The present study aimed to estimate genetic diversity using different types of molecular markers. Multiple mitochondrial fragments and nuclear microsatellite markers were selected for the study. The mitochondrial control region (also referred to

as mitochondrial d-loop) is highly useful for estimating genetic variation, population structure and evolutionary history owing to its high mutation rate (Brown et al., 1979; Castro et al., 1998). Although, mitochondrial control regions are highly suitable for population genetics studies other mitochondrial region/genes are also frequently used for genetic assessment studies and to compare previous findings (Table 2.1). The nuclear microsatellite markers are the first choice of marker for addressing a range of questions pertaining to population genetics such as genetic diversity, relatedness, population genetic structure, genetic differentiation, gene flow, effective population size, bottleneck, and mating system (Allendorf et al., 2010; Ellegren & Galtier, 2016)

Polymerase Chain Reactions (PCR) were carried out in 20 μ L volumes containing 2 μ L (10–20 ng) of the DNA template, 2 μ L of 10X DreamTaq buffer, 0.2 mM of each dNTP, 3 pmol of each primer and 0.1 μ L (0.5 units per reaction) of DreamTaq DNA polymerase (ThermoFisher Scientific) to amplify the fragments (Appendix 2). 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 visualized 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 (Appendix 3).

Table 2.1: Molecular genetics utility of different mitochondrial genes/regions.
The size of the asterisk signifies the useability of the region/gene in each category.

Region/Gene		Phylum	Class	Order	Family	Genus	Species	Population
rDNA	12S	*	*	*	*	*		
	16S				*	*	*	
Protein Coding Genes	ND1			*	*	*	*	*
	ND2			*	*	*	*	*
	COI			*	*	*	*	*
	COII			*	*	*	*	*
	CYT B			*	*	*	*	*
	CR					*	*	*

Source: (Hwang & Kim, 1999)

The selection and screening of the nuclear microsatellite markers (loci) were achieved in two stages. First, only gharial-specific microsatellite loci described by Jogayya et al., (2013) were listed and selected for screening. Eleven loci were chosen out of the 18 gharial-specific microsatellite loci that were described based on the number of alleles (≥ 4), allelic range (400), and heterozygosity (0.50). Only six of the eleven loci were found to be polymorphic in gharial samples after initial screening and temperature standardization.

Table 2.2: Primer sequence used to amplify partial mitochondrial *cytb* (Meganathan et al., 2009) and control region (CR) (Oaks, 2011).

Region	Primer	
	Name	Sequence (5'-3')
CR	L15637	GCATAACACTGAAAATGTTAAYATGG
	H16258	CTAAAATTACAGAAAAGCCGACCC
<i>cytb</i>	CP14715	TGAGGAGCAACCGTAATTACCAACCT
	CP15546	TCTGTCTTACAAGGCCAGTGCTTT

Since a panel of six loci exhibits lower statistical power to answer various population genetics questions in a later stage, an exhaustive literature search was done to identify cross-species microsatellite loci described across families Alligatoridae, Crocodylidae, and Gavialidae. A total of 424 previously described microsatellite loci were found and listed based on successful cross-species transferability, allele diversity, heterozygosity, polymorphic information content, and allele range. A panel of sixteen cross-species loci developed in eight different crocodylian species was selected for screening (Appendix 4). 12 of them were based on successful cross-species transferability seen in other crocodylian species, and the remaining four loci were selected based on the number of alleles ≥ 4 , H_o and $H_e \geq 0.5$, $PIC \geq 0.5$ and allelic range < 300 bp. Each of the selected pairs of microsatellite loci was screened with mugger samples. In total, a panel of 42 microsatellite loci was screened in gharial and 29 loci in mugger samples to obtain seven and ten polymorphic loci for population genetic

assessment. Finally, 232 gharial and 116 mugger samples were genotyped across seven and ten polymorphic loci. Each sample was genotyped in replicates (n=3) to obtain reliable multilocus genotypes using non-invasive samples following a multiple-tube approach (Miquel et al., 2006).

Polymerase Chain Reactions were performed in 10 μ L reaction volumes containing 5 μ L of 2x QIAGEN Multiplex PCR master mix, 2 μ L of 5x Q-solution (QIAGEN Inc., Germany), and the labelled forward primer at 0.15 μ M and the reverse primer at 0.15 μ M and 2 μ L of DNA template. The thermal profiles included an initial denaturation at 95 °C for 15 minutes, followed by 35 cycles at 94 °C for 40 seconds, 56-62 °C for 60 seconds, 72 °C for 60 seconds and a final extension of 72 °C for 30 minutes. The amplified products were genotyped using the GeneScan 500 LIZ dye size standard (Applied Biosystems, California, United States) in 3500XL Genetic Analyzer (Applied Biosystems, California, United States). The alleles were scored using GeneMarker v2.7.4 (SoftGenetics Inc., Pennsylvania, United States) with combined automated scoring and visually validated.

2.3.4 GENOTYPING QUALITY

Consensus genotypes were obtained for the three replicates, and the quality index for each locus genotyped was estimated following Miquel et al., (2006). If the genotype did not match the consensus due to any errors such as no amplification, allelic dropout, or false allele, the repeat was scored as “0,” and if the genotype was the same as the consensus, the repeat was scored as “1” (Miquel et al., 2006). The average amplification success was calculated as a percentage of positive PCR amplification.

The Frequency of genotyping errors due to allelic dropout (ADO) and false allele (FA) was estimated following (Broquet & Petit, 2004), null allele frequency was estimated using FreeNa (Chapuis & Estoup, 2007), and occurrences of large allelic dropout were estimated using Micro-Checker v2.2.3 (Van Oosterhout et al., 2004). Samples with a quality index above 0.67 per locus (identical genotypes in two replicates out of three replicates) and a mean quality index across loci above 0.75 were considered for downstream analyses.

2.3.5 MITOCHONDRIAL DNA VARIATION

All the sequences generated in this study were aligned using the CLUSTAL W algorithm in BioEdit v7.2.6 (Hall, 1999). The aligned sequences and associated electropherograms were checked manually. Sequence polymorphism/variations were confirmed by re-sequencing and considered when the base Phred quality score (Q) was greater than 20. Phred quality score is represented by 'Q.' It is used to indicate the measure of base quality in DNA sequencing. The value of Q is an integer representation of the probability p that the associated base call is incorrect. e.g., a Q value of 10 indicates there is a probability of 1 false base call in 10 bases sequenced (90%), and a Q value of 20 indicates an incorrect base call probability of 1 in 100 bases (99%).

Summary statistics, including the number of haplotypes (h), haplotype (gene) diversity (hd), and nucleotide diversity (Pi), were calculated using DnaSPv6.12 (Librado & Rozas, 2009). Additionally, 72 complete mitochondrial genomes

belonging to 23 crocodylian species were analysed to understand the genetic diversity of extant crocodylian species.

2.3.6 NUCLEAR MICROSATELLITE VARIATION

The probability of identity $P_{ID-biased}$ and $P_{ID-sibs}$ was calculated using Gimlet v1.3.3 (Genetic Identification with MultiLocus Tags) (Valière, 2002). The summary statistics (the number of alleles per locus, observed heterozygosity, expected heterozygosity, and inbreeding coefficient) were estimated using GenAlEx v6.0 (Peakall & Smouse, 2012), and deviations from Hardy–Weinberg equilibrium (HWE) for each locus using Bonferroni Corrections in Cervus v3.0.7 (Kalinowski et al., 2007).

2.4 RESULTS

A total of 232 gharials and 138 mugger samples were collected from the nesting sites along the Chambal River were collected for genetic assessment (Table 2.3).

Table 2.3: Detail of gharial and mugger samples collected for genetic diversity estimation in the Chambal River (T=Tissue and M=chorioallantoic membrane).

Species	River	No. of nesting sites	Samples collected
<i>Gavialis gangeticus</i>	Chambal	16	232 (T=65; M=167)
<i>Crocodylus palustris</i>	Chambal	4	138 (T=22, M=110)

2.4.1 MITOCHONDRIAL DNA VARIATION

A total of 103 gharial samples and 60 mugger samples were used to estimate the genetic variation at two mitochondrial regions. The mitochondrial CR sequences (n=103) in gharial were invariable with a single haplotype, while mugger CR sequences (n=51) had two haplotypes (mean $\pm$ SD) ($hd = 0.039 \pm 0.037$; $Pi = 0.00007 \pm 0.00007$). Gharial *cytb* sequences (n=103) revealed three haplotypes ($hd = 0.146 \pm 0.00207$; $Pi = 0.00022 \pm 0.00007$), while mugger sequences (n=60) revealed two haplotypes ($hd = 0.066 \pm 0.043$; $Pi = 0.00012 \pm 0.00008$) (Figure 2.1 and 2.2).

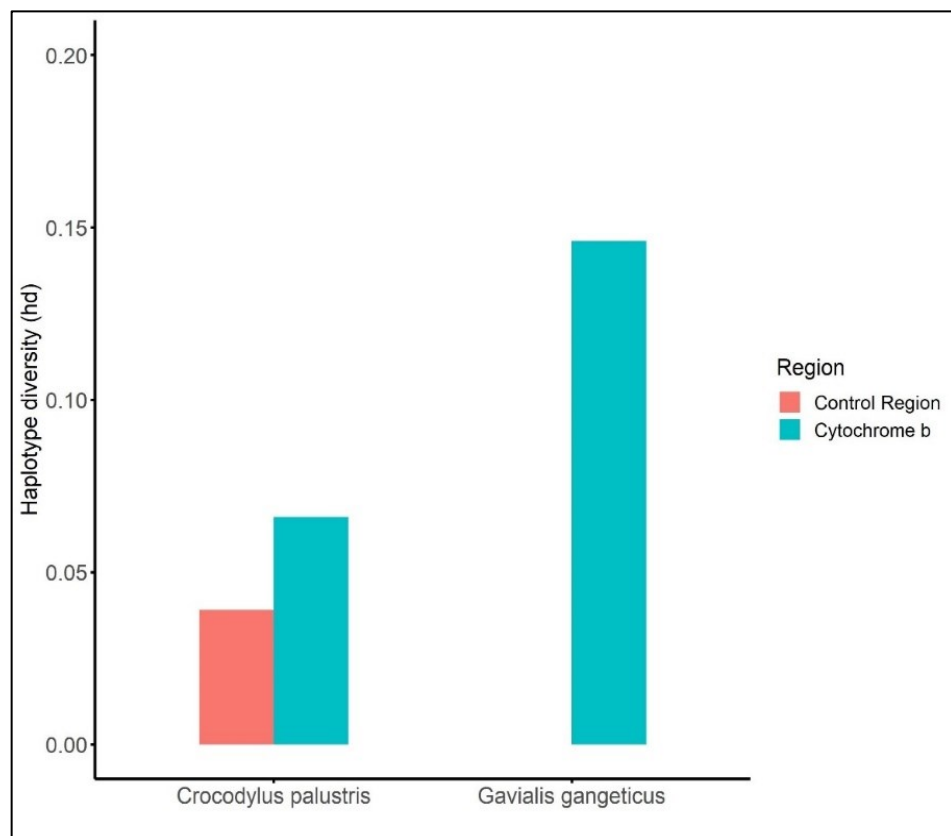


Figure 2.2: Graphical representation of haplotype diversity (hd) estimated using two partial mitochondrial fragments (*cytochrome b* and control region) in gharial (*Gavialis gangeticus*) and mugger (*Crocodylus palustris*).

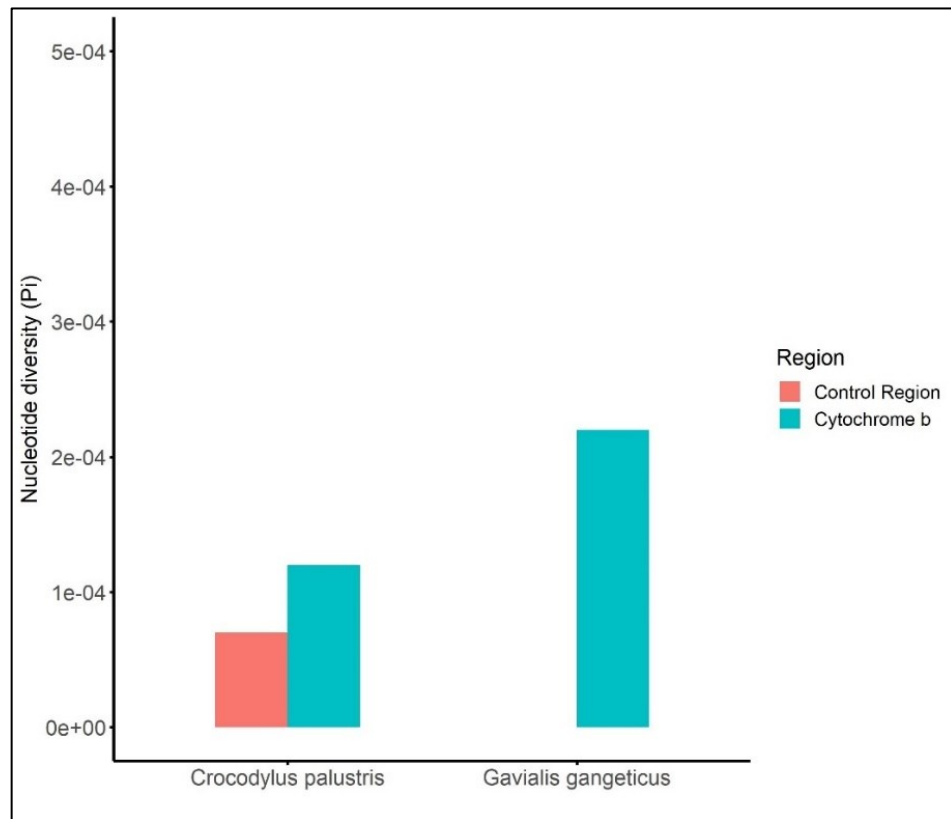


Figure 2.3: Graphical representation of nucleotide diversity (Pi) estimated using two partial mitochondrial regions (*cytochrome b* and control region) in gharial (*Gavialis gangeticus*) and mugger (*Crocodylus palustris*).

The complete mitochondrial genome analysis of 23 recognized crocodylian species using 72 sequences does not yield any association with IUCN conservation status or population size and distribution of the species (Figure 2.3). In comparison, *C. palustris* (n=4) showed higher genetic variation than *G. gangeticus* (n=3) (Table 2.4).

Table 2.4: A comparative chart of genetic diversity estimated using 72 complete mitochondrial genomes of 23 recognized crocodylian species. Number of sequences (N), the total number of sites (S), polymorphic or variable sites (P), the number of haplotypes (h), haplotype diversity (hd), and nucleotide diversity (Pi).

Species Name	N	S	P	h	Hd (mean $\pm$ SD)	Pi (mean $\pm$ SD)
<i>Alligator mississippiensis</i>	3	15897	14	2	0.667 $\pm$ 0.314	0.00059 $\pm$ 0.00028
<i>Alligator sinensis</i>	2	15897	14	2	1.0 $\pm$ 0.5	0.00088 $\pm$ 0.00044
<i>Caiman crocodilus</i>	3	16419	515	2	0.667 $\pm$ 0.314	0.02091 $\pm$ 0.00986
<i>Caiman latirostris</i>	2	16398	9	2	1.0 $\pm$ 0.5	0.00055 $\pm$ 0.00027
<i>Caiman yacare</i>	2	16392	2	2	1.0 $\pm$ 0.5	0.00012 $\pm$ 0.00006
<i>Melanosuchus niger</i>	2	15760	86	2	1.0 $\pm$ 0.5	0.00546 $\pm$ 0.0023
<i>Palaeosuchus palpebrosus</i>	4	14933	25	3	0.833 $\pm$ 0.22	0.00089 $\pm$ 0.00037
<i>Palaeosuchus trigonatus</i>	3	15945	157	2	0.667 $\pm$ 0.314	0.00656 $\pm$ 0.00309
<i>Crocodylus acutus</i>	3	16883	48	2	0.667 $\pm$ 0.314	0.0019 $\pm$ 0.00089
<i>Mecistops cataphractus</i>	4	15755	43	3	0.833 $\pm$ 0.222	0.00138 $\pm$ 0.00071
<i>Crocodylus intermedius</i>	3	16335	9	2	0.667 $\pm$ 0.314	0.00037 $\pm$ 0.00017
<i>Crocodylus johnstoni</i>	3	16844	84	2	0.667 $\pm$ 0.314	0.00332 $\pm$ 0.00157
<i>Crocodylus mindorensis</i>	3	16931	97	2	0.667 $\pm$ 0.314	0.00382 $\pm$ 0.0018
<i>Crocodylus moreletii</i>	3	16816	652	2	0.667 $\pm$ 0.314	0.02585 $\pm$ 0.01219
<i>Crocodylus niloticus</i>	4	16313	1034	3	0.833 $\pm$ 0.222	0.03285 $\pm$ 0.01407
<i>Crocodylus novaeguineae</i>	3	16306	1	2	0.667 $\pm$ 0.314	0.00004 $\pm$ 0.00002
<i>Crocodylus palustris</i>	4	16418	179	3	0.833 $\pm$ 0.222	0.00566 $\pm$ 0.00198

<i>Crocodylus porosus</i>	4	16904	233	2	0.667±0.314	0.00919±0.00413
<i>Crocodylus rhombifer</i>	3	16377	50	2	0.667±0.314	0.00204±0.00096
<i>Crocodylus siamensis</i>	3	16805	1428	2	0.667±0.314	0.05665±0.0267
<i>Osteolaemus tetraspis</i>	6	16233	1683	5	0.933±0.122	0.0353±0.0214
<i>Gavialis gangeticus</i>	3	17020	55	2	0.667±0.314	0.00215±0.00102
<i>Tomistoma schlegelii</i>	2	16883	37	2	1.0±0.5	0.00227±0.00113

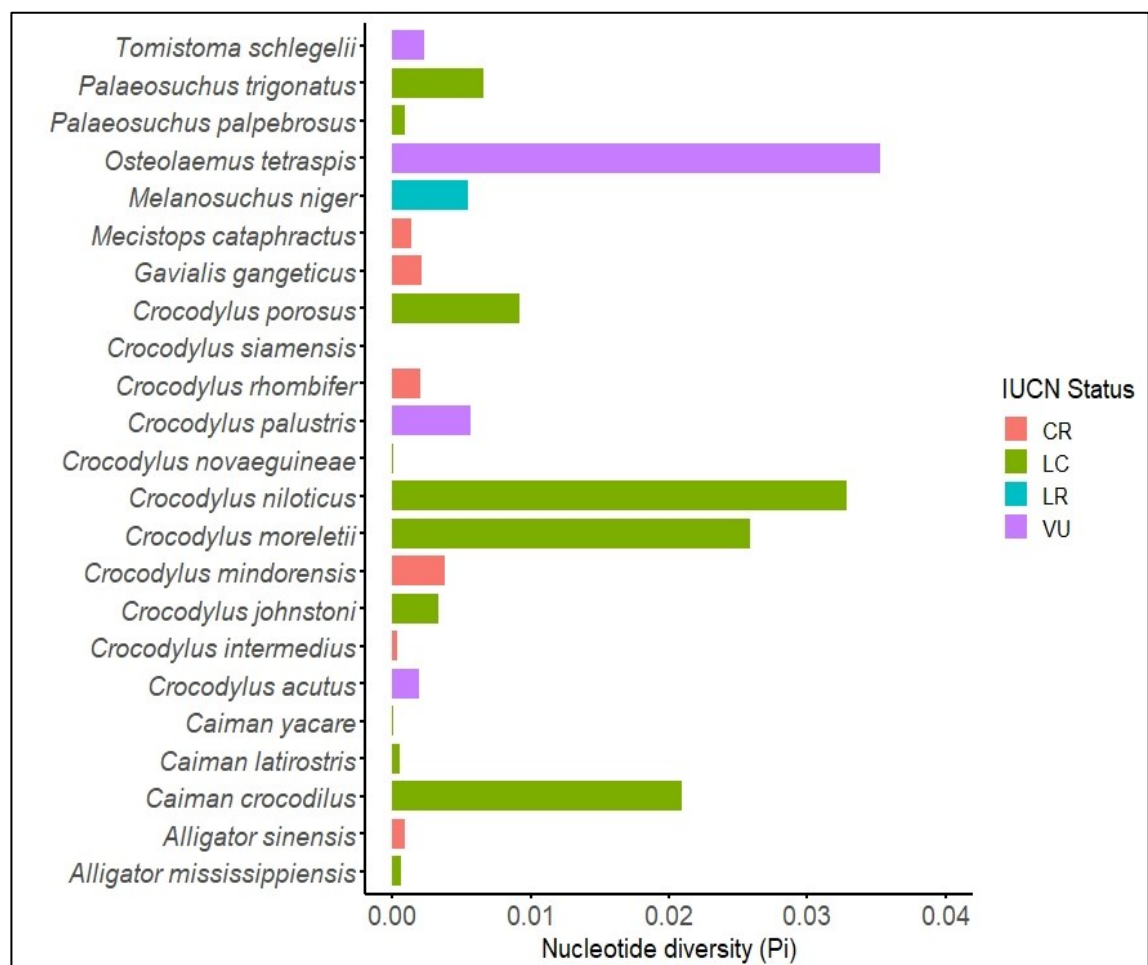


Figure 2.4: Graphical representation of nucleotide diversity (Pi) estimated across 23 crocodylian species.

2.4.2 GENOTYPING QUALITY

The amplification success of the polymorphic loci (Gharial=7 and Mugger=10) was 96.09%, and the quality index across the seven polymorphic loci was 0.86 ± 0.01 (mean $\pm$ SE) in gharial samples. The error rate per locus was below 15%, and null allele frequency was below 5% in gharial samples (Table 2.5). The amplification success across ten polymorphic loci in mugger samples was 88.61%, and the quality index was 0.93 ± 0.008 . The error rate per locus was below 10%, and null allele frequency was below 5% in mugger samples (Table 2.5). 228 gharial samples and 81 mugger samples were genotyped at seven and ten polymorphic loci, respectively.

Table 2.5: Amplification success and error rates calculated for seven polymorphic loci in gharial samples.

Locus	Amplification Success (%)	Quality Index	Error rate	Null Allele
G13_7	93.65	0.88	0.12	0.02
Cj16	93.53	0.89	0.11	0
G13_2	97.63	0.9	0.10	0
G13_5	97.45	0.83	0.17	0
G13_8	95.87	0.82	0.18	0
G13_14	95.9	0.85	0.15	0.02
G13_16	98.62	0.86	0.14	0
Mean	96.09	0.86	0.14	0.01
SE	0.74	0.01	0.01	0.004

Table 2.6: Amplification success and error rates calculated for seven polymorphic loci in gharial samples.

Locus	Amplification Success (%)	Quality Index	Error rate	Null allele
CpPSSR14	86.83	0.95	0.05	0.049
CpPSSR12	97.12	0.92	0.08	0.000
CUD68	84.36	0.88	0.12	0.000
4HDZ27	94.65	0.92	0.08	0.000
4HDZ391	92.59	0.90	0.10	0.000
G13_2	85.60	0.94	0.06	0.000
G13_14	85.19	0.95	0.05	0.024
G13_18	85.60	0.96	0.04	0.009
CpP203	85.60	0.95	0.05	0.000
CpP208	87.65	0.96	0.04	0.058
Mean	88.61	0.93	0.07	0.01
SE	1.44	0.008	0.008	0.007

2.4.3 NUCLEAR MICROSATELLITE VARIATION

The estimated cumulative $P_{ID-biased}$ was 1.74×10^{-3} , PID-sibs was 4.84×10^{-2} for gharial, and PID-biased was 9.93×10^{-8} $P_{ID-sibs}$ and 1.71×10^{-3} for the mugger. A total of 162 (71%) unique gharial individuals and 60 (74%) unique mugger individuals were identified using multilocus genotype data. The mugger sample from the DG

nesting site has only one individual genotype; therefore, it was excluded from further analysis.

The number of alleles per locus ranged between 2 to 7 in gharial and between 2 to 14 in mugger. The mean observed (H_o) and expected (H_e) heterozygosities were 0.42 ± 0.07 and 0.41 ± 0.06 in gharial and $H_o = 0.58 \pm 0.09$; $H_e 0.55 \pm 0.08$ in mugger. The genetic diversity estimates for gharial ($n=162$) and mugger ($n=59$) is summarised in Table 2.7 and Table 2.8, respectively

Table 2.7: Genetic diversity estimates of 162 individuals of gharial at seven microsatellite nuclear loci. The number of individuals (N), number of alleles per locus (N_a), allelic richness (A_r), observed heterozygosity (H_o), expected heterozygosity (H_e), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS).

Locus	N	N_a	H_o	H_e	HWE
G13_7	160	2	0.31	0.33	NS
Cj16	158	2	0.27	0.27	NS
G13_2	162	2	0.54	0.50	NS
G13_5	153	3	0.24	0.23	NS
G13_8	159	7	0.74	0.67	NS
G13_14	160	3	0.42	0.42	NS
G13_16	160	2	0.44	0.44	NS
Mean		3	0.42	0.41	
SE		0.69	0.07	0.06	

Table 2.8: Genetic diversity estimates of 60 individuals of mugger at ten microsatellite nuclear loci. The number of individuals (N), number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS), not done (ND), * significant at $P<0.05$.

Locus	N	Na	Ho	He	HWE
CpPSSR14	58	3.0	0.397	0.439	NS
CpPSSR12	60	2.0	0.051	0.050	ND
CUD68	58	5.0	0.724	0.705	NS
4HDZ27	60	3.0	0.780	0.621	*
4HDZ391	60	14.0	0.898	0.866	NS
G13_2	60	4.0	0.780	0.610	NS
G13_14	60	4.0	0.373	0.369	ND
G13_18	60	2.0	0.475	0.488	NS
CpP203	60	13.0	0.881	0.832	*
CpP208	60	2.0	0.407	0.493	NS
Mean		5.20	0.58	0.55	
SE		1.42	0.09	0.08	

2.5 DISCUSSION

The genetic assessment of the two freshwater crocodylians in the Chambal River using nuclear microsatellite and mitochondrial markers reveals a low level of genetic variability. Both the mitochondrial region *viz.* *cytb* and control region showed a low level of sequence polymorphism across species. The mitochondrial control region, which is otherwise highly variable, was invariable in 103 gharial samples, and only two haplotypes were observed in 60 mugger samples. The invariable mitochondrial control region in gharial samples was unusual and first documented in any crocodylian species. The presence of extremely low variation at a mitochondrial hypervariable region is surprising because studies have reported considerably high sequence polymorphism in other crocodylian species (Luck et al., 2012; Ray et al., 2004; Rossi et al., 2020; Russello et al., 2007; van Asch et al., 2019). The *cytb* region was also found to have low sequence polymorphism; nonetheless, the variability was higher in the gharial samples compared to the mugger samples. Moreover, the overall nuclear diversity estimated using nuclear microsatellite markers varied in gharial and mugger. The estimated indices of genetic diversity, some alleles per locus, heterozygosities, and the mean number of alleles were higher in the mugger.

In wild crocodylian populations, a low level of variation at mitochondrial and nuclear markers is not rare. Similar levels of genetic variation have been reported in other crocodylians, including *Alligator sinensis* (Zhu et al., 2009), *Alligator mississippiensis* (Glenn et al., 1998), *Caiman yacare* (Ojeda et al., 2017), *Crocodylus siamensis* (Yu et al., 2011), *Crocodylus mindorensis* (Hinlo et al., 2014), *Crocodylus moreletii* (Dever et al., 2002; Mcvay et al., 2008), and *Crocodylus palustris* (Campos et al., 2018), as

well as in other vertebrate species that have experienced demographic bottlenecks (Garner et al., 2005). Moreover, such low levels of mitochondrial nucleotide diversity are possibly explained by the known history of a severe population decline, genetic bottleneck, and assisted population recovery with a small number of founder individuals in these species. The genetic diversity estimates heterozygosity and allelic richness in the Chambal gharial population is lower than in captive gharial populations ($H_o = 0.92 \pm 0.02$; $H_e 0.65 \pm 0.02$; $A_r=5.5 \pm 0.5$) using the same set of microsatellite loci (Jogayya et al., 2013) is of concern.

The genetic diversity of a species is influenced by its geographic range, abundance, demography, and life-history traits. Narrow-ranging, less abundant, and long-lived species with demographically challenged population history tend to have lower genetic diversity than widely distributed, abundant species with stable demographic history (Eckert et al., 2008; Ellegren & Galtier, 2016). The low abundance, narrow distribution range, complex life-history traits, and unstable demography of the gharial may have resulted in the low level of genetic diversity. In contrast, a moderate level of genetic diversity in the mugger population may be due to the species' life-history traits, demographic history, and rapid population recovery.

The most straightforward mechanism that species adapt to avoid inbreeding is dispersal. However, crocodylians are known to have strong philopatry. Hence, it is implausible to use dispersal as an inbreeding counter mechanism. Other species that have evolved to adapt mechanisms to avoid inbreeding are kin avoidance, multiple paternity, and delayed maturation or reproductive suppression (Moore & Ali, 1984; Pusey & Wolf, 1996).

In conclusion, low polymorphism was observed in species-specific and cross-species microsatellite loci; it remains crucial to develop novel microsatellite loci for population genetic studies. I acknowledge the inference of the study might have been influenced by the inclusion of closely related individuals from each nesting site and a limited number of polymorphic markers in gharial. This is the first genetic assessment of the wild gharial and mugger population in India, which will serve as a baseline for future genetic monitoring to assess the recovery of these species and research.

CHAPTER 3

POPULATION GENETIC STRUCTURE. DIFFERENTIATION AND GENE-FLOW

3.1 SUMMARY

The complex interaction between natural and anthropogenic factors shapes genetic structure in a natural population. Specifically, species life-history traits such as dispersal, reproductive strategies, and human induce fragmentation are key in shaping the genetic structure of a population. The presence of genetic structuring in a species can be a result of the species own traits or could be enforced due to human-induced fragmentation and loss of connectivity. Both of these disrupt the gene-flow resulting in population divergence, often leading to increased rates of inbreeding, a loss of heterozygosity, and the fixation of deleterious alleles. Such effects are of a magnitude high in a small population and species showing limited dispersal and or philopatry. Crocodiles are shown to have strong philopatry. Hence it is expected to have strong population differentiation. Analyses of population genetic structure using nuclear microsatellite markers suggested weak inter-population structure genetic structuring and the absence of intra-population structure in gharial. In contrast, the analysis suggests genetic structure at both inter- and intra-population levels in mugger. Genetic structure within small geographic regions corresponding to river basins or riverscapes is not rare in crocodylians. Such patterns are observed due to species-specific traits such as philopatry.

3.2 INTRODUCTION

Species in the wild are naturally predisposed to spatial and/or temporal structuring due to clustered and uneven resource distribution (Turner, 1989). Synergetic interaction of multiple biotic and abiotic factors, such as life-history, social organization, behavioral, ecological requirements, and habitat dynamics, determine the extent of structuring (Matsuzawa et al., 2016; Moore et al., 2008). Processes like gene-flow, natural selection, and genetic drift are thought to play important roles in shaping the genetic structure of natural populations (Eckert et al., 2008). “Dispersal is any movement of individuals that has potential consequences for gene-flow across space (Saastamoinen et al., 2018)”. Among these factors, gene-flow plays a central role in determining spatial structuring in species, where long dispersing (presence or high of gene-flow) species are likely to exhibit weak spatial structuring, whereas low or non-dispersing (low or no gene-flow) species display strong structuring (Slatkin, 1987). In the absence of gene-flow, the population becomes disjunct and is expected to lose genetic diversity due to genetic drift and increase the risk of inbreeding, reducing the population's resilience and adaptability (Allendorf et al., 2010).

In the recent past, anthropogenic stressors such as habitat fragmentation apart from species life history have rapidly suppressed and become a key force in determining population genetic structure (Radosavljević et al., 2015; Waters et al., 2020). The impact of fragmentation is likely to vary between species (Bailey et al., 2010; Ewers & Didham, 2006), which acts primarily by disrupting the connectivity between populations, restricting the gene-flow (dispersal), and increasing the genetic divergence over time (Moore et al., 2008). The impact of disrupted connectivity due

to fragmentation is much higher in longitudinally connected systems such as riverine species than in terrestrial species. The ultimate consequence of fragmentation may sometimes lead to a species's local or regional extinction (Davis et al., 2018; Saunders et al., 1991). The effects of fragmentation are either scaled as less severe or overlooked often because of the late-occurrence of their impact, which often takes generations to become evident. In concordant with the small population paradigm, the severity of fragmentation is higher in species living in small numbers and varies with species' life-history traits. Dispersal-induced gene flow is thought to reduce genetic divergence between populations and is likely to slow or impede local adaptation in species (Arendt, 2015). In contrast, gene-flow is a major force that can reduce the extinction probability by increasing diversity, reducing population divergence, and eliminating other negative consequences of improvised genetic diversity (Blyth et al., 2020). Hence, population genetic structure, genetic differentiation, and gene-flow can thus have positive and well as negative consequences for populations

Species exhibit unique life-history traits known as site fidelity or philopatry. Fidelity or philopatry has been reported to result in genetic structuring (Arbogast et al., 2022; Lee et al., 2007), and studies involving different taxonomic groups have linked philopatry to fine-scale genetic structuring over successive generations, e.g., (Miller et al., 2010; Storz, 1999). Despite being used interchangeably in practice, these terms are distinguishable. The term 'philopatry' refers to a species' tendency to establish itself or return to the birth sites. While species' tendency to disperse and subsequently establish itself in a new area and return to the same site/location time and again is called 'site-fidelity' (Reed & Oring, 1993). The choice to be fidelity has

advantages as well as disadvantages. The most important and known benefits are a better understanding of resources to maximize resource utilization and safety from predators. Consequently, on the other side, species showing strong philopatry and limited dispersal ability are highly expected to induce genetic structuring, sometimes leading to increased inbreeding due to the low number of contributing parents within the area (Lance et al., 2009; Rönkä et al., 2021). Such observations are common in turtles and crocodylians, which exhibit strong philopatry (Lance et al., 2009; Rossi et al., 2020).

Crocodylians are among a group of taxa that predominantly display philopatry behavior. The behavior can vary among species and populations, and some crocodylian species have been known to disperse over long distances with varied dispersal abilities (Baker et al., 2022; Rossi et al., 2020; Tucker et al., 1998). The extent of dispersal can be influenced by various factors, including population density, food availability, and environmental conditions, and the ability to disperse can vary among different life stages, with juveniles being more likely to disperse than adults (Lang, 1987; Singh, 1985). The gharial (*Gavialis gangeticus*), the most aquatic of all crocodylians, and their ecological specialist nature and low mobility are likely to have strong genetic structures. And living in a regulated riverine system is likely to induce a strong genetic structure (Vashistha et al., 2021). While mugger, which is ecological generalist, presence of no social structure, they are less likely to be structured. The study of population genetic structure enables, and gene-flow can provide vital information on species' complex life-history traits such as migration and philopatry. This information is particularly useful for managers in managing landscapes to

improve connectivity among natural populations as this provides a variety of ecological and genetic benefits to take appropriate conservation measures to maintain genetic diversity and alleviate the negative consequences of fragmentation. The present chapter focused on population genetic structure to address the following research questions (a) What is the level of genetic structuring in gharial and mugger (a) Do the Chambal and Girwa River populations have their genetic signatures? (b) If so, what is the level of differentiation? and (c) Did the demographic decline suffered by gharial populations in the last century also induce a genetic bottleneck?

3.3 METHODOLOGY

In this study, population genetic structure, genetic differentiation, and gene-flow of two freshwater crocodiles managed in two riverscapes *viz.* Chambal and Girwa rivers were inferred using nuclear microsatellite markers.

3.3.1 SAMPLE COLLECTION

In addition to the samples collected for genetic assessment in the Chambal River (Chapter 2), a total of 116 gharial samples collected from three nesting sites and 16 mugger samples from one nesting site along the Girwa river within Katarniaghat Wildlife Sanctuary were genotyped to infer the population genetic structure (Table 3.1).

Table 3.1: Detail of gharial and mugger samples collected for population genetics structure and gene-flow (T=Tissue and M=chorioallantoic membrane).

Species	River	No. of nesting sites	Samples genotyped
Gharial	Chambal	16	232 (T=65; M=167)
(<i>Gavialis gangeticus</i>)	Girwa	3	116 (T=11; M=105)
Mugger	Chambal	4	122 (T=22, M=100)
(<i>Crocodylus palustris</i>)	Girwa	1	16 (T=0, M=16)

3.3.2 DNA EXTRACTION

Total genomic DNA was extracted from tissue and chorioallantoic membrane using the phenol-chloroform method (Sambrook et al., 1989) (Appendix 1). Cotton swabs were used when the chorioallantoic membrane was unable to separate from eggshells or failed to recover DNA.

3.3.3 GENOTYPING QUALITY

Consensus genotypes were obtained for the three replicates, and the quality index for each locus genotyped was estimated following Miquel et al. (2006). If the genotype did not match the consensus due to any errors such as no amplification, allelic dropout, or false allele, the repeat was scored as “0,” and if the genotype was the same as the consensus, the repeat was scored as “1” (Miquel et al., 2006). The average amplification success was calculated as a percentage of positive PCR amplification. The Frequency of genotyping errors due to allelic dropout (ADO) and false allele (FA) was estimated following (Broquet & Petit, 2004), null allele frequency was estimated

using FreeNa (Chapuis & Estoup, 2007) and occurrences of large allelic dropout were estimated using Micro-Checker v2.2.3 (Van Oosterhout et al., 2004). Samples with a quality index above 0.67 per locus (identical genotypes in two replicates out of three replicates) and a mean quality index across loci above 0.75 were considered for downstream analyses.

3.3.4 NUCLEAR MICROSATELLITE VARIATION

Summary statistics (alleles per locus, heterozygosities-observed and expected, and inbreeding coefficient) were estimated for Girwa River samples using GenAlEx v6.0 (Peakall & Smouse, 2012) before performing population genetic analysis. In addition, each locus was tested for departure from Hardy–Weinberg equilibrium (HWE) using Bonferroni correction in Cervus v3.0.7 (Kalinowski et al., 2007).

3.3.5 POPULATION GENETICS STRUCTURE AND GENETIC DIFFERENTIATION

Two approaches were used to infer the population structure. First, a Bayesian approach implemented in Structure v2.3.4 was used to infer population genetic structure (Pritchard et al., 2000). Structure is a systematic model-based clustering approach that uses allele frequencies at each locus to infer the population structure. Initially, the analysis was performed without providing the sampling location as *a priori*, and then it was performed with the sampling location as *a priori*. Except for providing sampling location as *a priori*, the analysis was performed using the same set of parameters. The analysis was performed considering a maximum of 10 clusters (K), and for each K , 10 iterations were run under the admixture with correlated allele frequencies model. The simulation model using location *a priori* performs well when

no clear signal of a structure is detected or when there is low genetic differentiation, limited loci, or a limited number of samples are used to infer population genetic structure (Hubisz et al., 2009). The simulations were run for 1,00,000 burn-in and 10,00,000 Markov chain Monte Carlo iterations (MCMC). The optimum number of K was inferred using the likelihood distribution $L(K)$ and the delta K , which were estimated in the web version of Structure Harvester, v0.6.94 (Earl & VonHoldt, 2012). The assignment plot was prepared using Distruct v1.1 (Rosenberg, 2004). Second, a discriminate analysis of principal component (DAPC), which is a multivariate non-model-based approach, was used to identify and describe genetic clusters between two riverscape populations (Jombart et al., 2010). The optimal number of the clusters was estimated based on the lowest associated Bayesian Information Criterion (BIC). The analysis was performed using the *adgenet* (Jombart et al., 2010) in the R package.

Genetic differentiation was calculated between Chambal and Girwa. Three genetic differentiation indices, F_{ST} , G'_{ST} (fixation), and Jost's D (allelic differentiation) were used to estimate the genetic differentiation. The indices were calculated using *strataG* (Archer et al., 2017) implemented in the R package. The estimates were calculated using 1000 bootstrap iterations.

3.3.6 GENE-FLOW

Similarly, the gene-flow was estimated between Chambal and Girwa populations for both gharial and mugger. BayesAss v3.0 was used to estimate the recent migration rate (m) (Wilson & Rannala, 2003). Three independent simulations run with varying seed numbers were selected to perform the simulation using 10^7 MCMC iterations and 10^6 burn-in periods.

3.4 RESULTS

Overall, a total of 348 gharial samples (Chambal=232 and Girwa=116) were genotyped at seven polymorphic loci, and 138 mugger samples (Chambal=122 and Girwa=16) to infer the population genetic structure, genetic differentiation, and gene-flow.

3.4.1 GENOTYPING QUALITY

The amplification success across polymorphic loci was 96.09 % for gharial and 88.52 % for mugger in Chambal River and 95.38 % for gharial, and 84.80 % for mugger in Girwa samples. The quality index for gharial samples across seven polymorphic loci was 0.86 ± 0.01 (mean $\pm$ SE) in Chambal, and 0.89 ± 0.01 in Girwa samples, and the quality index calculated across ten polymorphic loci in mugger samples was 0.93 ± 0.008 in Chambal and 0.85 ± 0.004 in Girwa River. The overall error rate per locus across polymorphic loci was below 15%, and null allele frequency was below 5% across species and populations analysed. None of the population showed any occurrence of large allele dropout (Table 3.2 and 3.3).

Table 3.2: Summary of the amplification success and error rates frequency calculated for gharial samples genotyped at seven polymorphic loci. CH=Chambal River and GR=Girwa River.

Locus name	Amplification success (%)		Quality Index		Error rate per locus		Null allele frequency	
	CH	GR	CH	GR	CH	GR	CH	GR
G13_7	93.65	98.86	0.88	0.88	0.120	0.120	0.02	0.00
Cj16	93.53	84.86	0.89	0.85	0.110	0.150	0.00	0.00

G13_2	97.63	95.73	0.9	0.91	0.100	0.090	0.00	0.00
G13_5	97.45	--	0.83	--	0.170	--	0.00	--
G13_8	95.87	97.14	0.82	0.93	0.180	0.070	0.00	0.05
G13_14	95.9	97.13	0.85	0.92	0.150	0.080	0.02	0.05
G13_16	98.62	98.56	0.86	0.87	0.140	0.130	0.00	0.00
Mean	96.09	95.38	0.86	0.89	0.14	0.11	0.01	0.02
SE	--	--	0.01	0.01	0.01	0.01	0.004	0.009

Table 3.3: Summary of the amplification success and error rates frequency calculated for mugger samples genotyped at ten polymorphic loci. CH=Chambal River and GR=Girwa River.

Locus name	Amplification success (%)		Quality Index		Error rate per locus		Null allele frequency	
	CH	GR	CH	GR	CH	GR	CH	GR
CpPSSR14	86.83	84.02	0.95	0.92	0.050	0.078	0.0486	0.0029
CpPSSR12	97.12	90.28	0.92	0.85	0.077	0.146	0.0000	0.0001
CUD68	84.36	85.10	0.88	0.89	0.116	0.108	0.0000	0.0020
4HDZ27	94.65	89.65	0.92	0.87	0.078	0.128	0.0000	0.0000
4HDZ391	92.59	84.02	0.90	0.81	0.101	0.187	0.0000	0.0000
G13_2	85.60	82.40	0.94	0.91	0.059	0.091	0.0000	0.0000
G13_14	85.19	84.30	0.95	0.94	0.047	0.056	0.0241	0.0030
G13_18	85.60	82.20	0.96	0.92	0.042	0.076	0.0094	0.0012
CpP203	85.60	81.20	0.95	0.91	0.051	0.095	0.0000	0.0010

Locus name	Amplification success (%)		Quality Index		Error rate per locus		Null allele frequency	
	CH	GR	CH	GR	CH	GR	CH	GR
CpP208	87.65	84.80	0.96	0.93	0.037	0.066	0.0582	0.0070
Mean	88.52	84.80	0.93	0.90	0.066	0.103	0.0140	0.0017
SE			0.01	0.01	0.01	0.01	0.01	0.001

3.4.2 NUCLEAR MICROSATELLITE VARIATION

A total of 66 (57 %) unique gharial individuals and 8 (50 %) unique mugger individuals were identified using multilocus genotype data. The number of alleles per locus ranged between 2 to 5 in gharial and between 1 to 7 in mugger. The mean observed (H_o) and expected (H_e) heterozygosities were 0.42 ± 0.08 and 0.42 ± 0.09 in gharial and $H_o = 0.63 \pm 0.09$; $H_e = 0.55 \pm 0.07$ in mugger. The genetic diversity estimates for gharial ($n=66$) and mugger ($n=8$) are summarised in Table 3.4 and 3.5, respectively.

Table 3.4: Genetic diversity estimates of 66 gharial individuals (Girwa River) six polymorphic microsatellite nuclear loci. Number of individuals (N), number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS), not done (ND).

Locus	N	Na	Ho	He	HWE
G13_7	66	2	0.50	0.49	NS
Cj16	66	2	0.03	0.03	NS
G13_2	66	2	0.56	0.48	NS
G13_8	66	5	0.53	0.63	NS
G13_14	66	3	0.42	0.52	NS
G13_16	66	2	0.47	0.36	NS
Mean		2.67	0.42	0.41	
SE		0.49	0.08	0.09	

Table 3.5: Genetic diversity estimates of eight individuals (Girwa River) of mugger at ten microsatellite nuclear loci. Number of individuals (N), number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), Hardy-Weinberg equilibrium (HWE), not significant after Bonferroni Correction (NS), not done (ND).

Locus	N	Na	Ho	He	Significance
CpPSSR14	8	3.000	0.625	0.570	NS
CpPSSR12	8	1.000	0.000	0.000	ND
CUD68	8	5.000	0.875	0.727	NS

Locus	N	Na	Ho	He	Significance
4HDZ27	8	3.000	0.875	0.586	NS
4HDZ391	8	7.000	0.875	0.813	NS
G13_2	7	3.000	0.857	0.541	NS
G13_14	8	3.000	0.500	0.531	NS
G13_18	8	2.000	0.625	0.492	NS
CpP203	8	6.000	0.750	0.781	NS
CpP208	8	2.000	0.375	0.430	NS
Mean	7.900	3.500	0.636	0.547	
SE	0.100	0.601	0.090	0.073	

3.4.3 POPULATION GENETIC STRUCTURE AND GENETIC DIFFERENTIATION

The Bayesian approach implemented in Structure v2.3.4 identified two ($K = 2$) optimum numbers of genetic clusters in gharial samples using both models (with and without location priors) (Appendix 4). Only a small proportion of individuals were assigned to specific groups, and a large proportion of individuals were found to be in admixed form, i. e., individuals cluster membership coefficient (q) was below 0.75 for any of the inferred (Figure 3.1 and Table 3.6). Cluster membership coefficient (q) ≤ 0.75 were considered as admixed.

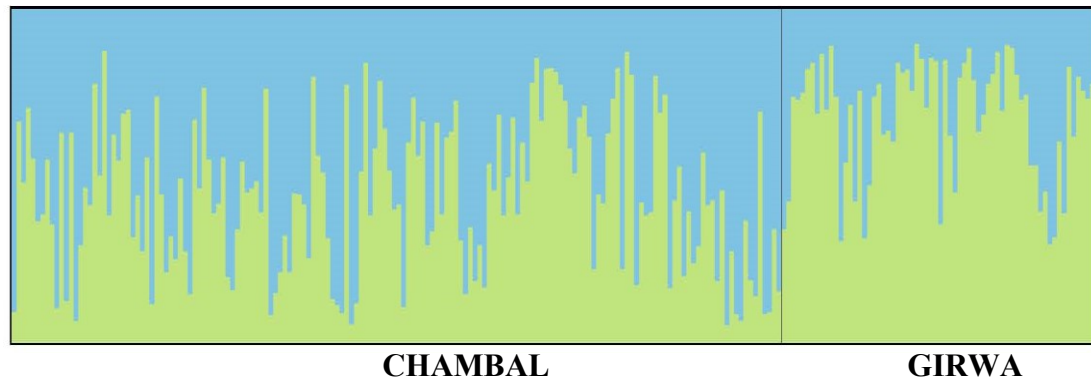


Figure 3.1: Population genetic structure of 228 gharial individuals derived using a Bayesian clustering approach implemented in STRUCTURE without location priors. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster.

Table 3.6: Number and percent of gharial individuals assigned to each genetic cluster inferred using Bayesian clustering approach implemented in STRUCTURE

Population	Cluster		
	I	II	Admixed
Chambal	18 (11 %)	37 (23 %)	107 (66 %)
Girwa	30 (45 %)	0	36 (55 %)

Further, analysis using location information as *a priori* (locprior model) assigned all Girwa samples to cluster-I, with an average proportion of the membership, $q = 0.97$, and a larger portion (73%) of Chambal individuals as admixed and only 23 % to cluster-II (Figure 3.2).

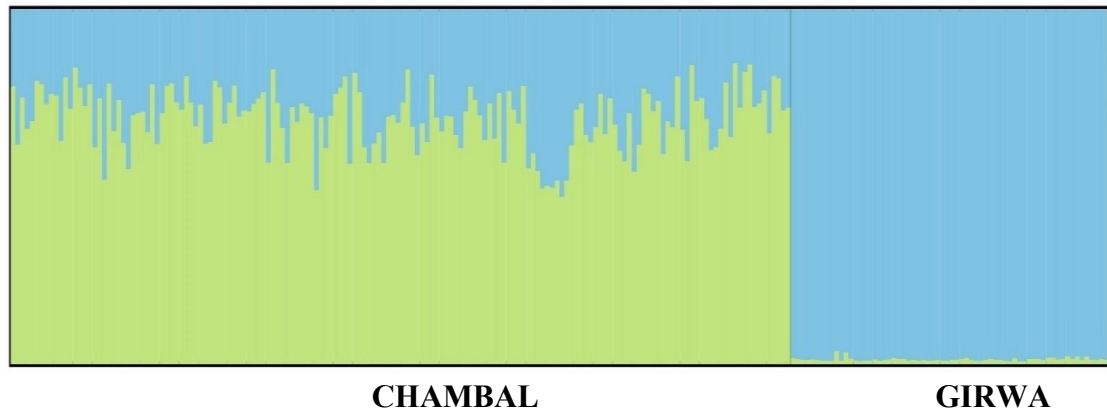


Figure 3.2: Population genetic structure of 228 gharial individuals derived using a Bayesian clustering approach implemented in STRUCTURE with location priors. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster.

Table 3.7: Number and percent of gharial individuals assigned to each genetic cluster inferred using Bayesian clustering approach implemented in STRUCTURE

Population	Cluster		
	I	II	Admixed
Chambal	43 (27 %)	0	119 (73 %)
Girwa	0	66 (100 %)	0

The multivariate approach DAPC identified 20 genetic clusters associated with the lowest BIC (Bayesian information criterion) (Appendix 2). All the identified clusters ($K = 20$) showed an overlapping pattern with no clear structuring of samples (Figure 3.3). No intra-population genetic structure was observed in gharial samples.

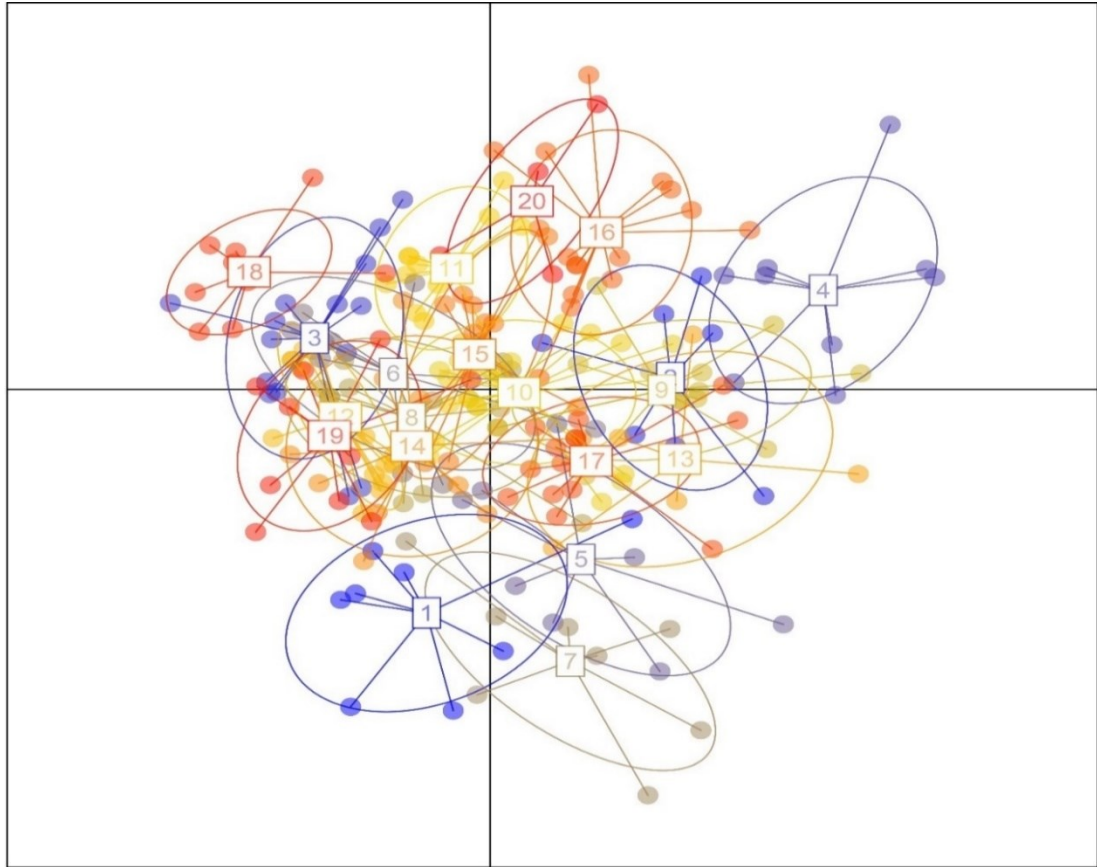


Figure 3.3: Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 228 gharial individuals. This scatterplot shows the first two principal components of the DAPC. The individuals are shown as a point.

The Bayesian approach implemented in Structure v2.3.4 identified two ($K = 4$) optimum numbers of clusters in mugger samples using both models (with and without location priors) (Appendix 1). Wherein all Girwa samples were assigned to a single cluster with an average proportion of membership (q) of 0.95, and Chambal samples were assigned to multiple clusters (34% - cluster-I, 8% -cluster-II, 19%-cluster-III, 31%-cluster-IV and 8%-admixed) indicating the presence of intra-population structure in Chambal samples (Figure 3.4 and Table 3.4). The DAPC identified six clusters

(K=6) using the Bayesian Information Criterion (BIC) (Appendix 4), segregating them into three units (Figure 3.5).

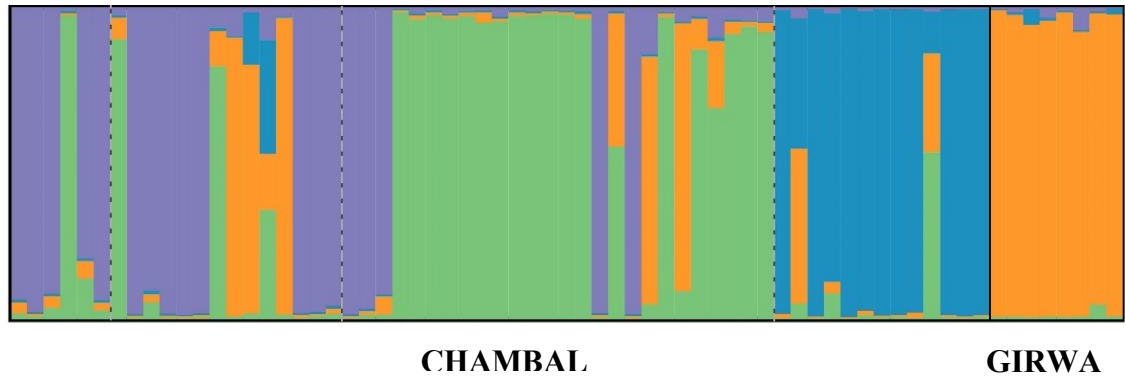


Figure: 3.4: Population genetic structure of 67 mugger individuals derived using a Bayesian clustering approach implemented in STRUCTURE without location priors. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster.

Table 3.8: Number and percent of mugger individuals assigned to each genetic cluster inferred using the Bayesian clustering approach implemented in STRUCTURE

Population	Cluster				
	I	II	III	IV	Mixed
Chambal	20 (34 %)	5 (8 %)	11 (19 %)	18 (31 %)	5 (8 %)
Girwa	0	8 (100 %)	0	0	0

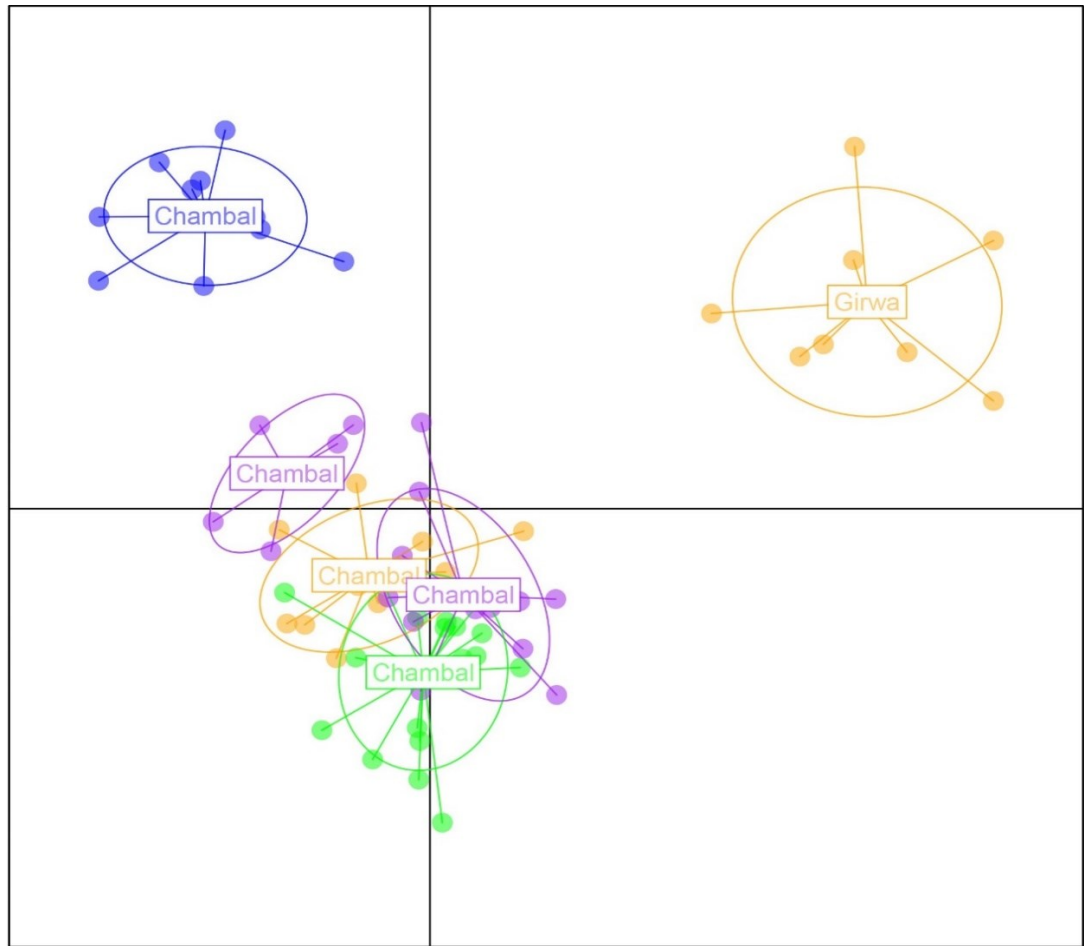


Figure 3.5: Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 67 gharial individuals. This scatterplot shows the first two principal components of the DAPC. Dots represent individuals.

Further, to explore intra-population genetic structure in mugger population, both analyses were performed only with Chambal samples. The Bayesian clustering analysis suggests the presence of three ($K = 3$) clusters inferred by the delta K estimates (Appendix 4). All individuals sampled from PA were assigned to cluster I with an overall proportion of membership (q) equal to 0.975. 92 % of individuals sampled from TG were assigned to cluster-III with q equal to 0.901 and the remaining 8 % to cluster-

I. The individuals sampled from BR and NG were assigned to multiple clusters (BR; 50 % - cluster I, 29 % - cluster II and 3% - admixed and NG; 23 % - cluster I, 73 %-cluster II, and 4 % - admixed) (Figure 3.6 and Table 3.5).

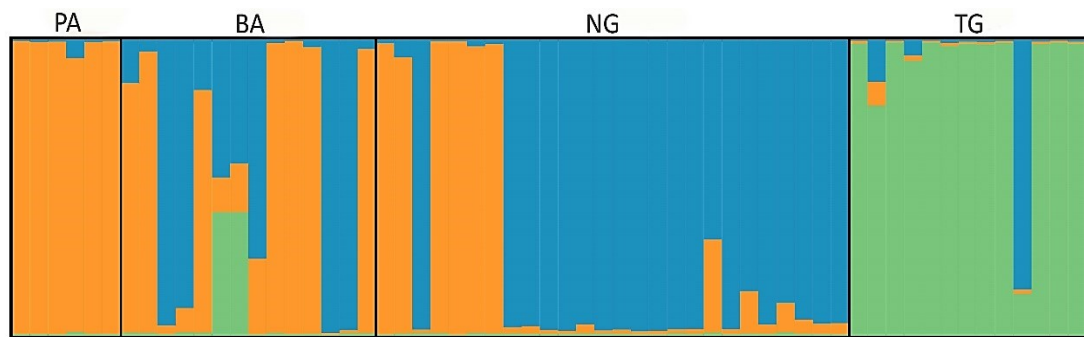


Figure 3.6: Population genetic structure of 59 mugger individuals derived using a Bayesian clustering approach implemented in STRUCTURE without location priors. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster.

Table 3.9: Number and percent of mugger individuals assigned to each genetic cluster inferred using the Bayesian clustering approach. PA-Pali, BR-Baroli, NG-Nadigaon and TG-Tigri

Nesting sites	Cluster			
	I	II	III	Mixed
PA	6 (100 %)	0	0	0
BR	7 (50 %)	4 (29 %)	0	3 (21%)
NG	6 (23 %)	19 (73 %)	0	1 (4 %)
TG	1 (8 %)	0	12 (92 %)	0

DAPC identified five clusters (K=5) using the Bayesian Information Criterion (BIC) (Appendix 4), segregating them into three units (Figure 3.7). Overall, both analyses showed three genetic clusters within the sampled stretch (I-PA, II-BR/NG, and III-TG).

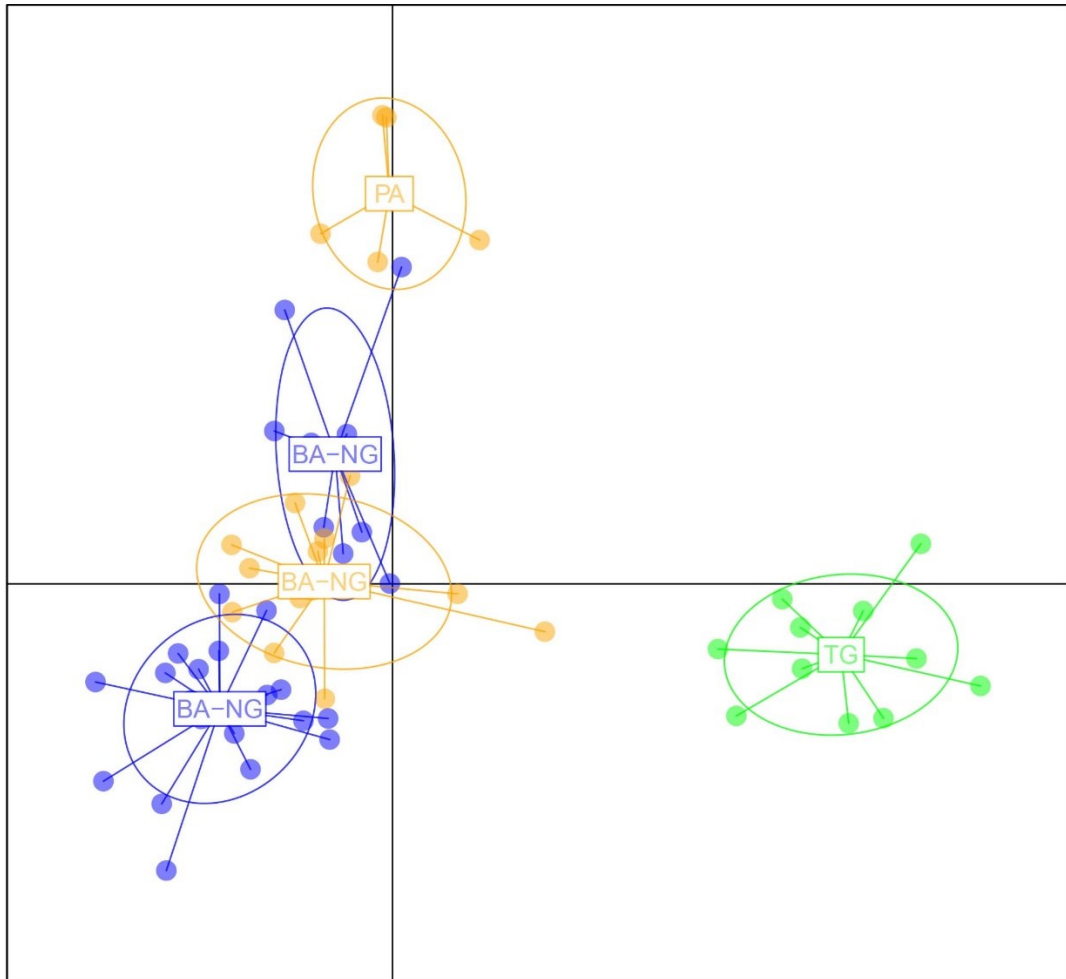


Figure 3.7: Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 59 mugger individuals. This scatterplot shows the first two principal components of the DAPC. Dots represent individuals.

In gharial samples, the genetic differentiation measures were low but significant as derived using both the fixation indices: $F_{ST} = 0.039$ ($P < 0.05$); $G'_{ST} =$

0.058 ($P < 0.05$) and allelic differentiation index Jost's $D = 0.016$ ($P < 0.05$). In mugger samples, the genetic differentiation measures were relatively higher: $F_{ST} = 0.135$ ($P < 0.05$); $G'_{ST} = 0.104$ ($P < 0.05$), and allelic differentiation index Jost's $D = 0.193$ ($P < 0.05$).

Intra-population genetic differentiation in Chambal mugger samples was significant ($P < 0.05$) between all the sites except for BR and NG. The F_{ST} was estimated highest between PA-TG sites at 0.289 ($P < 0.05$) and lowest between PA-BR sites at 0.096 ($P < 0.05$). The G'_{ST} also showed a similar pattern, where the highest was estimated between PA-TG sites at 0.202 ($P < 0.05$) and the lowest between PA-BR sites at 0.064 ($P < 0.05$).

Table 3.10: Genetic differentiation between nesting sites in Chambal River. The value below the diagonal represents F_{ST} , and below represents the p-value. * represents a significant p-value. PA- Pali, BR-Baroli, NG-Nadigaon and TG-Tigri

Nesting sites	PA	BR	NG	TG
PA	--	0.096*	0.149*	0.289*
BR	0.003	--	0.011	0.159*
NG	<0.001	0.116	--	0.178*
TG	<0.001	<0.001	<0.001	--

Table 3.11: Genetic differentiation between nesting sites in Chambal River. The value below the diagonal represents G'_{ST} , and below represents the p-value. *= represents a significant p-value. PA- Pali, BR-Baroli, NG-Nadigaon and TG-Tigri.

Nesting sites	PA	BR	NG	TG
PA	--	0.064*	0.102*	0.202*
BR	0.003	--	0.008	0.113*
NG	<0.001	0.115	--	0.128*
TG	<0.001	<0.001	<0.001	--

Table 3.12: Genetic differentiation between nesting sites in Chambal River. The value below the diagonal represents Jost's D , and below represents the p-value. *= represents a significant p-value. PA- Pali, BR-Baroli, NG-Nadigaon and TG-Tigri.

Nesting sites	PA	BR	NG	TG
PA	--	0.093*	0.152*	0.277*
BR	0.005	--	0.013	0.113*
NG	<0.001	0.115	--	0.203*
TG	<0.001	<0.001	<0.001	--

3.4.4. GENE-FLOW

The recent (contemporary) migration rate (m) in gharial was 0.128 (95% CI: 0–0.272) from Chambal to Girwa River and 0.021 (95% CI: 0–0.058) from Girwa to Chambal (Figure 4). The recent migration rate in mugger was 0.035 (95% CI: 0–0.096) from

Chambal to Girwa and 0.007 (95% CI: 0–0.019), which is lower than the migration rate observed in gharial individuals (Figure 3.8 and 3.9). The recent migration among the nesting sites within Chamba River was highest between Baroli-Pali, and a low migration rate was observed between other sites (Figure 3.10)

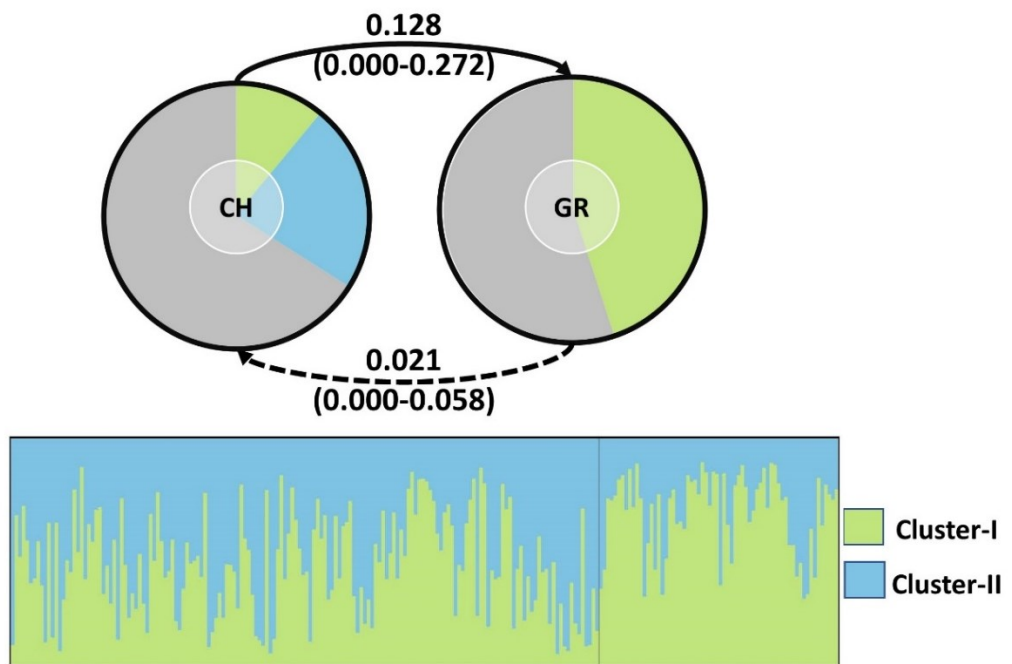


Figure 3.8: Recent migration rate (m) in gharial individuals between Girwa and Chambal populations was estimated using the Bayesian approach implemented in BayesAss. The values in the parenthesis represent a 95% confidence interval. CH=Chambal and GR=Girwa River.

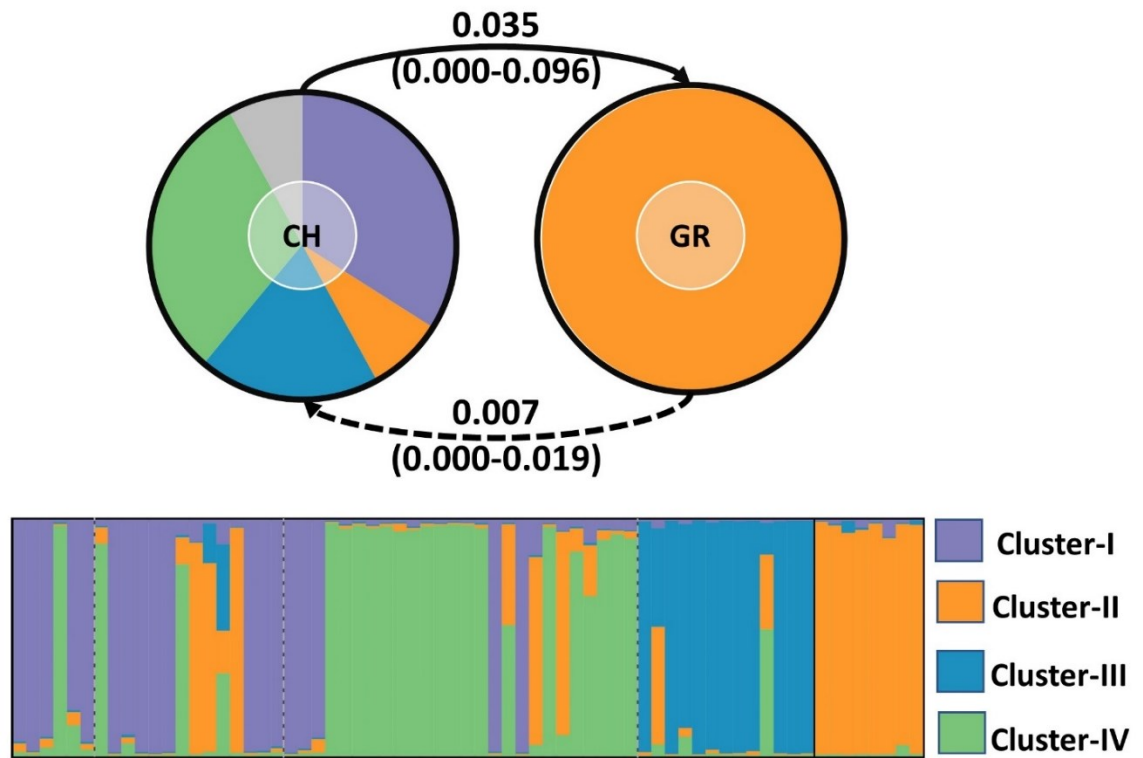


Figure 3.9: Recent migration rate (m) estimated in mugger individuals between Girwa and Chambal populations using the Bayesian approach implemented in BayesAss. The values in the parenthesis represent a 95% confidence interval. CH=Chambal and GR=Girwa River.

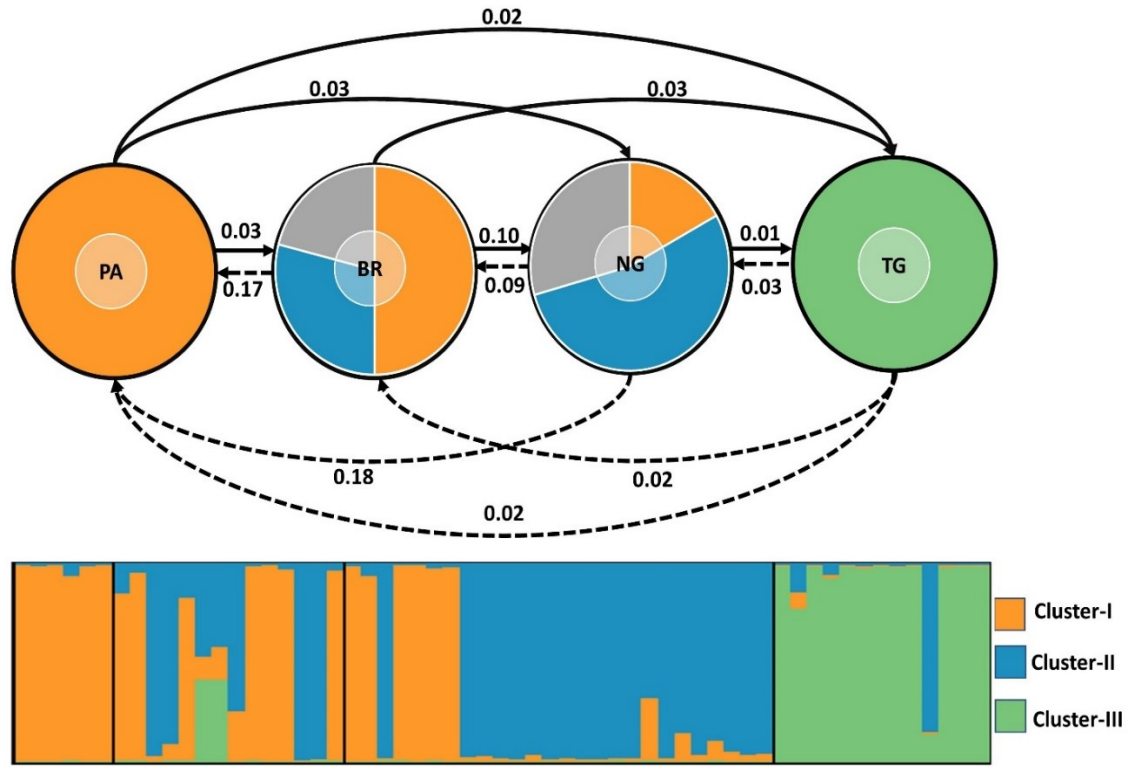


Figure 3.10: Recent migration rate (m) estimated within Chambal River mugger individuals using the Bayesian approach implemented in BayesAss. The values in the parenthesis represent a 95% confidence interval. PA=Pali, BR=Baroli, NG-Nadigaon and TG=Tigri.

3.5 DISCUSSION

The population genetic structure in two sympatric crocodylian species was inferred using nuclear microsatellite markers. The information on population genetic structure, genetic differentiation, and gene-flow provides crucial information on connectivity between populations and complex species life-history traits such as philopatry and dispersal, which are essential for the conservation and management of species and contributes to the maintenance of genetic diversity in populations (Saastamoinen et al., 2018). Considering the rapid increase in anthropogenic stressors

and their negative impacts on the species' long-term persistence, information on these complex phenomena, which are key for maintaining genetic diversity, is necessary for a population characterized by low genetic diversity. Reptiles, including crocodylian, turtles, and lizards, displays their unique social structure, philopatry, and complex dispersal ability that directly or indirectly guides and shapes the genetic structure (Baker et al., 2022; Moore et al., 2020). Recent studies have shown factors such as interspecific interactions, e.g., hybridization, and anthropogenic stressors, e.g., poaching, habitat destruction, fragmentation, and planned and unplanned translocation of individuals, determine the species' genetic structure (Rossi et al., 2020).

Shallow genetic structure with a low level of differentiation observed in gharial between two riverscapes, at least 1500 km apart, is unique. In the first instance, this contrasts with species' life-history traits and stows away the likely impact of fragmentation in the riverine system. Though most of the crocodylians are characterised as philopatric, the report of long-distance migration of crocodylians, including gharial, are not unusual (Grigg & Kirshner, 2015; Rossi et al., 2020). As a result, genetic structuring is expected in these species, and some species have even been reported to exhibit genetic structuring over small geographic scales (riverscape and river basin) (Rossi et al., 2020; van Asch et al., 2019).

Unlike gharial, the mugger population showed a clear pattern of geographically (riverscape) structured population (Figures 3.4 and 3.5). Further, the fine-scale investigation of the intra-population structure in the Chambal mugger samples revealed a clear pattern of upstream downstream (riverscape) genetic structuring in the mugger population (Figures 3.6 and 3.7). The identified genetic cluster followed a

pattern where individuals from upstream nesting sites were assigned to two clusters with no clear geographical-genetic separation. However, individuals from the downstream nesting site (Tigri) were assigned to a single genetic cluster with a high membership coefficient $q > 0.9$. The limited long-distance movement probably explains the observed pattern of genetic structuring mugger population (Singh, 1985) and might be due to the species life-history traits, such as philopatry, observed in crocodiles (Cunningham et al., 2016; van Asch et al., 2019). As an ecological generalist, the mugger can appropriate resources in each habitat without dispersing across longer distances in search of optimal basking, nesting, and foraging habitats. Hence, the presence of multiple genetic clusters within Chambal River sites may have been due to the limited movement of the individuals across sites. In a few cases, the spatial genetic structure is indistinct because a few individuals of the same population are assigned different genetic clusters.

Dissimilar population genetic structures observed in these species could be attributed to the difference in their life-history traits or the possible impact of human-assisted conservation translocation. Considering previous reports of strong genetic structuring in crocodylians mainly attributed to species life-history traits rather than human-mediated factors. Unlike gharial, there is no record of the inter-mixing of individuals and inter-population release of mugger individuals in these rivers. The presence of riverscape-segregated genetic structure in mugger is therefore considered accurate and likely due to species life-history traits as these populations are growing naturally with the least conservation interventions. Presumably, the absence of population genetic structure in gharial and low differentiation in gharial is likely due

to human-assisted gene-flow, which might have occurred during the release of gharial individuals (Kelly & Phillips, 2016; Weeks et al., 2015). The findings are of particular interest because two sympatric species were assessed for the presence of genetic structure. This provides information on species-specific traits and the influence of ‘head-start’ assisted conservation translocation. Due to the unavailability of the old distributional records of mugger in the north Indian rivers, it is anticipated that they must have experienced different selection parameter founder effects rather than the bottleneck, which may yield similar results and are often difficult to differentiate. These are descriptive analyses based on allele frequency developed in a model of a discrete population of equal sample size and equal immigrants. Hence, a difference in sample size across the nesting sites could have led to a biased estimate of differentiation.

Restricted dispersal reduces the effective size of a population and can erode genetic diversity in species. There is considerable interest in managing landscapes to improve connectivity among natural populations as this provides various ecological and genetic benefits, including the potential for natural genetic rescue in genetically repressed populations (Jangjoo et al., 2016). The estimates of the gene-flow analysis are consistent and supported by findings of population genetic structure and genetic differentiation analysis. For gharial, relatively high inter-population (Chambal $\longleftrightarrow$ Girwa) gene flow was observed, and the direction of gene-flow was high from Chambal to Girwa and low from Girwa to Chambal (Figure 3.8). The observed pattern could be because Chambal being the largest population since the initiation of the conservation program, a higher number of eggs were sourced from

Chambal, and reared individuals were released in Girwa River. Whereas low inter-population (Chambal $\leftrightarrow$ Girwa) gene-flow (Figure 3.9). The intra-population (within Chambal) gene-flow was also low between most of the nesting sites except Pali $\leftarrow$ Baroli, Baroli $\leftarrow$ Nadigaon and Pali $\leftarrow$ Nadigaon, which showed a high level of gene-flow (Figure 3.10). The findings of the reverse (Upstream $\leftarrow$ Downstream) direction of gene-flow in mugger were unexpected, given a unidirectional gene-flow, mostly Upstream $\rightarrow$ Downstream observed in the riverine system.

The observed pattern of gene-flow in mugger could be due to species colonizing new areas upstream of the river. The upstream migration could be due to several factors, such as suitable habitat, availability of prey, and an increase in the overall population size. The upstream of the river (Pali-Dangbasai) is characterised by rocky habitat interspersed with sand. Hence, mugger migrating upstream could be due to an increase in their population size and species occupying the most suitable habitat. The pattern of gene-flow in this species can provide important information about the dispersal and colonizing abilities of the mugger crocodile and can help us better understand the processes that drive their distribution and evolution.

CHAPTER 4

INCIDENCE OF MULTIPLE PATERNITY IN GHARIAL AND MUGGER

4.1 SUMMARY

Species opt and adapt to various complex and cryptic life-history traits to buffer the negative consequence of reduced genetic diversity. Multiple paternity is one such cryptic behavior reported to be common in reptiles and has been linked to offering multiple genetics and social benefits. In the present study, mugger samples ($n=22$) from the Nadigaon nesting site in the Chambal River were analysed for the incidence of multiple paternity. Combined paternity exclusion (PE) and the Probability of detecting multiple paternity (PrDM) were estimated to test the robustness of the microsatellite loci. The overall PE for 10 loci (when neither parent genotype was known as 0.94 and PrDM ranged between 0.60 – 0.99 (when 10 offspring analysed per clutch). The results confirmed the presence of multiple paternity using both allele count and stepwise progeny arrays method in mugger in the Chambal River. The incidence of multiple paternity in gharial could not be assessed due to low polymorphism in nuclear microsatellites. Multiple factors such as male density, low genetic diversity, and a high degree of inbreeding are known to act as selective forces that are likely to trigger multiple paternity in reptiles, specifically crocodylians. The information on the mating system of crocodylians is vital to guide the ongoing conservation translocation program, and this will assist the management of the species captive breeding program and conservation efforts.

4.2 INTRODUCTION

Species in the natural system use various mating systems either as a mechanism to reduce the costly male-female encounter or to buffer and repress short and long-term negative consequences arising from complex social structures (Baker et al., 2022). Reptiles, including crocodylians, are known to show a complex hierarchical social-structural with a dominant alpha male controlling the group (Grigg & Kirshner, 2015). Such complex hierarchical social structures are known to erode genetic diversity and lower the species' adaptability (Ellegren & Galtier, 2016). Multiple paternity is a behavioral and compensatory mechanism that has often been observed in reptiles that contribute to lowering the negative consequence of low genetic diversity (Chesser & Baker, 1996; Sugg & Chesser, 1994; Wang et al., 2017). Multiple paternity occurs when a litter or clutch of eggs are shared by more than one male (Uller & Olsson, 2008). Species are often known to gain various benefits from opting for and adapting to such a mating system (Sugg & Chesser, 1994; Uller & Olsson, 2008). Successful adaptation of these strategies in species is assumed to increase genetic variability, fertilization, and effective population size (Chesser & Baker, 1996; Zajdel et al., 2019). Further, multiple paternity has been linked to multiple social and genetic benefits rather than just consequences of male-female encounters (Lee et al., 2007).

The mating system in crocodylians is known to be polygynous, with social groups dominated by alpha males (Doody et al., 2021; Lang, 1987). These dominant alpha males in *Alligator mississippiensis* have been observed interrupting courtships between females and subordinate males (Garrick & Lang, 1977). However, to what extent these subordinate males succeed in accomplishing mating has been researched.

The range-wide decline in the crocodylian population owing to overexploitation in the mid-20th century (Bustard, 1975; Grigg & Kirshner, 2015) is assumed to result in the loss of genetic diversity in many of the crocodylians across the globe (Amavet et al., 2008; Sharma et al., 2020, 2021). Studies assessing the genetic diversity in crocodylians have often reported a low level of genetic diversity measured in terms of low sequence variation, low allelic number, and heterozygosities (Aggarwal et al., 2014; Campos et al., 2018; Dever et al., 2002; Glenn et al., 1998; Hinlo et al., 2014; Luck et al., 2012; Ojeda et al., 2017; Ray et al., 2004; Rossi et al., 2020; Russello et al., 2007; Sharma et al., 2021; van Asch et al., 2019; Yu et al., 2011; Zhu et al., 2009), except a few studies in captivity, has moderate to high levels of genetic diversity (Jogayya et al., 2013). The demographic decline due to overexploitation is majorly attributed to the anthropogenic cause. However, a species' life history also contributes significantly to maintaining genetic diversity along with other forces. The loss of genetic diversity has been associated with reduced fitness and vulnerability to population depletion and a higher extinction risk (Allendorf et al., 2010; Reed & Frankham, 2003).

Hence, understanding the incidence of multiple paternity in sympatric crocodylian species that have suffered substantial population decline and subsequent recovery, which has shown different levels of genetic diversity, will help gain insight into such cryptic and complex biological processes to inform conservation decisions. These life-history traits are key determinants that regulate species' genetic diversity (Ellegren & Galtier, 2016). The study of such a cryptic and complex process in aquatic species is even more difficult when the whereabouts of the individuals in question are

difficult to address through field observations. However, recent advances in DNA-based molecular techniques as has become a tool of interest in answering various biological questions that are otherwise difficult to answer (Lewis et al., 2013; Sunnucks, 2000). The field observation has limitations on several Fronts where it is difficult to identify the actual mating partner. Hence studies based on microsatellites were the first to report the incidence of multiple paternity in crocodylians. Microsatellite-based paternity analysis is one such robust and recommended tool used to study the incidence of multiple paternity in reptiles, including crocodylians (Hu & Wu, 2010; Isberg, 2022; Lewis et al., 2013; Uller & Olsson, 2008; Zajdel et al., 2019)

So far, genetic studies of crocodylian mating systems have been conducted on the *Alligator mississippiensis* (Davis et al., 2001; Lance et al., 2009), *Alligator sinensis* (Hu & Wu, 2010), *Crocodylus moreletti* (Dever et al., 2002; Mcvay et al., 2008), *Caiman latirostris* (Amavet et al., 2008) and *Crocodylus porosus* (Isberg, 2022; Lewis et al., 2013). As often seen in several taxa such as mammals, birds, and reptiles, the dominant male controls the group, maintain territoriality, and is responsible for guarding the group and offspring (Uller & Olsson, 2008). Species adapt to various mechanisms to maintain or to increase the probability of surviving the offspring. Polyandry, where one female mate with multiple males, which often results in the involvement of multiple males in a single mating season, is one such mechanism that has been reported in reptiles that can help increase the survival of offspring (Amavet et al., 2008; Hu & Wu, 2010; Uller & Olsson, 2008).

Hence, a higher incidence of multiple paternity in gharial is hypothesized than in mugger. The study of mating systems is essential in conservation translocation, as

the removal of individuals involved in breeding may disrupt social systems and impact the population (Rowe & Hutchings, 2003) or reduce genetic diversity.

4.3 METHODOLOGY:

4.3.1 SAMPLE COLLECTION AND DNA EXTRACTION

During the post-hatching survey across the Chambal River, six complete clutches of gharial from two nesting sites and two complete clutches of mugger from one nesting site were collected for paternity analysis. The chorioallantoic membrane from all the eggs was separated and stored in absolute ethanol at room temperature and later at -20 °C in the laboratory for long-term storage. Cotton swabs were used when the chorioallantoic membrane was unable to separate from eggshells. The total genomic DNA for multiple paternity assessments was extracted using the phenol-chloroform method (Sambrook et al., 1989).

4.3.2 MICROSATELLITE SELECTION AND EFFECTIVENESS OF THE MARKERS

The efficacy of detecting multiple paternity is directly influenced by the number of offspring analysed per clutch, the number of putative fathers, sire-ratio, clutch size, and the degree of microsatellite polymorphism (Neff & Pitcher, 2002; Uller & Olsson, 2008). Although studies have assessed multiple paternity using a minimum of two microsatellite markers, the power to detect multiple paternity increases with the number of loci analysed (Conley & Gerrish, 2019). Previous paternity studies on crocodylians have used a minimum of four (Amavet et al., 2008; Hu & Wu, 2010) and five (Davis et al., 2001; Mcvay et al., 2008; Zajdel et al., 2019) six and above (Milián-García et al., 2016; Muniz et al., 2011; Rossi et al., 2016) loci

detect the presence of multiple paternity with adequate statistical power. A total of 42 species-specific and cross-species microsatellite loci were screened on a representative subset of samples collected across the nesting sites (Appendix 4).

The probability of exclusion (i.e., the ability of a set of loci to exclude a random individual who is not an actual parent) was estimated to assess the effectiveness of the loci in performing paternity analysis under three conditions both parent pair genotypes known, one parent genotypes known and neither parent genotypes are known on all loci and subset of loci exhibiting alleles per locus ≥ 4 . The probability of detecting multiple paternity (PrDM) was calculated on various sets of conditions; two males' contributions under equal (50/50), moderately skewed (67/33), and highly skewed (90/10), and three males' contributions under equal (33.3/33.3/33.3), moderately skewed (57/28.5/14.5) and highly skewed (80/15:5) with a different number of eggs per clutch from 5-50.

4.3.3 ANALYSIS OF MULTIPLE PATERNITY

We have used two approaches to detect the incidence of multiple in mugger samples, i) single locus minimum method – wherein diversity estimate of each of the nest were calculated. This is based on the criteria that offspring inherit one allele from each parent and that a clutch of full siblings can only display a maximum of 4 alleles at one given locus (Lotsander et al., 2021). If any clutches showing more than four alleles per locus were regarded as the presence of multiple paternity and (ii) Multilocus stepwise progeny arrays method, where parental genotypes are reconstructed when both parents' genotypes were unknown. The analysis was performed assuming single female and multiple males. The software GERUD 2.0 was used to calculate the

minimum number of males for each clutch (Jones, 2005). The software utilises a reconstructed genotype method based on all offspring multilocus genotypes. All single-locus genotypes are combined to create all possible multilocus parental genotypes. The minimum number of fathers required to explain the progeny array is estimated using an exhaustive sequential search.

4.4 RESULTS

Based on the criteria set for the selection of microsatellite loci for paternity analysis (allele per locus ≥ 4) a total of three microsatellite loci were selected in mugger (Table 4.1), while only one locus fulfilled the criteria in gharial of exhibiting four or more alleles per locus.

Table 4.1: List of species-specific and cross-species polymorphic microsatellite loci in gharial and mugger. * Loci selected for paternity analysis in the mugger.

Locus	Alleles per locus	
	Gharial	Mugger
G13_8	7	2
G13_5	3	--
G13_14*	3	4
G13_2*	2	4
G13_7	2	--
G13_16	2	--
Cj16	2	1
G13_18	--	2

CpSSR12	--	2
CUD68*	--	5
4HDZ27	--	3
CpSSR14	--	3
4HDZ391*	--	14
CpP203*	--	13
CpP208	--	2

The probability of exclusion (i.e., the ability of a set of loci to exclude a random individual who is not an actual parent) was estimated to assess the effectiveness of the loci to perform paternity analysis when neither parent genotypes were (7 loci= 0.496) in gharial and (10 loci=0.938; 5 loci=0.890) in mugger. The probability of exclusion was calculated on samples collected for genetics assessment across all loci (n=10) and a subset of loci (n=5) exhibiting alleles per locus ≥ 4 in mugger (Table 4.2). While due to a low number of loci than recommended (n=3) (Cifuentes Ovalle et al., 2006) with alleles per locus for ≥ 4 the exclusion probability was calculated across all loci (n=7) in gharial (Table 4.3). Hence, due to a low number (≥ 3) of polymorphic loci (allele per locus ≥ 4) than required and low combined exclusion probability, the parentage analysis could not be performed with gharial samples.

Table 4.2: Combined exclusion probability estimated to assess the effectiveness of microsatellite loci to perform parentage analysis in mugger. The exclusion probability was estimated for a complete set of loci.

Number of loci	Exclusion probabilities
One parent genotype is known with certainty, and one is unknown	
Complete set loci (10 loci)	0.992
Neither parent genotype is known	
Complete set loci (10 loci)	0.938
Parent pairs genotype in known	
Complete set loci (10 loci)	1.000

Table 4.3: Combined exclusion probability estimated to assess the effectiveness of microsatellite loci to perform parentage analysis in gharial.

Number of loci	Exclusion probabilities
One parent genotype is known with certainty, and one is unknown	
7	0.797
Neither parent genotype is known	
7	0.496
Parent pairs genotype in known	
7	0.934

The probability of detecting multiple paternity (PrDM) estimated was high when males' contribution was equal (50:50). The PrDM for two males contributing equally was 0.97, and for three males was 0.99 when 10 or more eggs per clutch were analyzed. While the estimate was lower for contribution skewed (90:10), two males were 0.60, and three males were 0.85 when 10 eggs per clutch were analyzed. The probability of detecting multiple paternity (PrDM) under different scenarios is depicted in Figure 4.1.

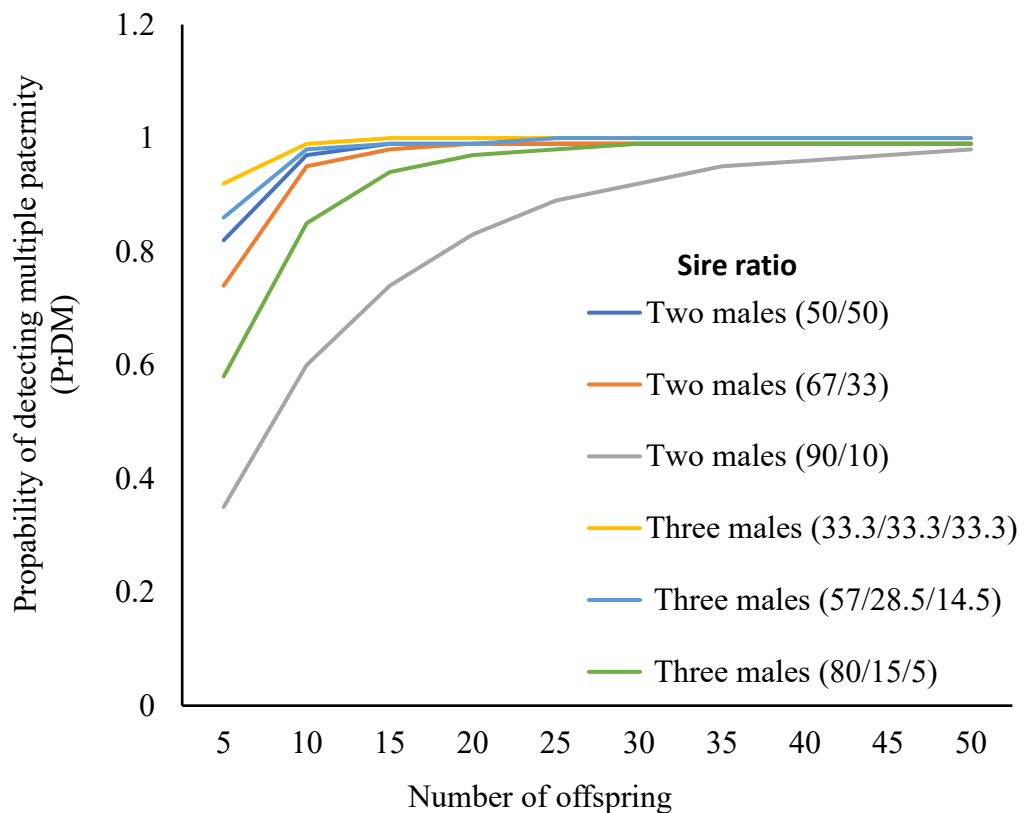


Figure 4.1: Probability of detecting multiple paternity (PrDM) for five microsatellite loci in mugger at a different number of putative males (sire ratio) and a number of offspring per clutch.

The paternity analysis was performed in two clutches of mugger eggs collected from the Nadigaon nesting site in the Chambal River. Both the nests analysed showed the incidence of multiple paternity (Table 4.3). The manual single locus minimum method suggests the contribution of at least two males in the second clutch, while the multilocus stepwise progeny arrays method performed in GERUD v2.0 inferred a minimum of two males' contribution in both the clutches analysed.

Table 4.4: Number of alleles per locus per nest. MF=Minimum number of fathers inferred using multilocus stepwise progeny arrays method

Clutches		Clutch 1		Clutch 2	
Locus		Allele	Frequency	Allele	Frequency
CUD68 (Clutch 1-3; Clutch 2-2)		162	0.35	162	0.21
		164	0.25	164	0.79
		168	0.40	--	--
		177	0.35	173	0.29
		185	0.35	175	0.33
4HDZ391 (Clutch 1-4; Clutch 2-5)		197	0.10	185	0.17
		203	0.20	203	0.17
		--	--	205	0.04
		310	0.45	310	0.25
G13_2 (Clutch 1-2; Clutch 2-3)		314	0.55	314	0.50
		--	--	326	0.25
		278	0.70	278	0.75

(Clutch 1-2; Clutch 2-2)	296	0.30	292	0.25
	269	0.40	265	0.17
CpP203	275	0.60	269	0.17
(Clutch 1-2; Clutch 2-4)	--	--	275	0.33
	--	--	277	0.33
MF	2		2	

4.5 DISCUSSION

The incidence of multiple paternity has been reported in a variety of vertebrate and invertebrate species (Akçay & Roughgarden, 2007; Arnqvist & Nilsson, 2000; Uller & Olsson, 2008), including lizards (Eizaguirre et al., 2007; Fitze et al., 2005; Laloi et al., 2004), turtles and tortoises (Crim et al., 2002; Fitzsimmons, 1998; Harry & Briscoe, 1988; Johnston et al., 2006; Pearse & Avise, 2001; Valenzuela, 2000) and are known to offer various direct and indirect benefits to the species (Uller & Olsson, 2008). The incidence of multiple paternity in crocodylian has been studied in *Alligator mississippiensis* (Davis et al., 2001; Zajdel et al., 2019), *Alligator sinensis* (Hu & Wu, 2010), *Crocodylus moreletii* (McVay et al., 2008), *Caiman latirostris* (Amavet et al., 2008) and *Crocodylus porosus* (Isberg, 2022; Lewis et al., 2013). Within taxa, the incidence of multiple paternity is known to vary between species and populations and is attributed to multiple factors (Uller & Olsson, 2008; Wang et al., 2017).

The results confirmed the presence of multiple paternity using both allele count and stepwise progeny arrays method in mugger in the Chambal River. Despite its low number in the 1970s, the mugger population grew steadily without much of the

conservation interventions, such as reinforcement of captive-bred or captive-reared individuals. A relatively high level of genetic diversity in mugger could be due to its ecological generalist nature and successful adaption to behavioral mechanisms like multiple paternity, which are known to increase genetic diversity in a species (Sugg & Chesser, 1994; Wang et al., 2017).

Studies also suggest that multiple factors are responsible for the incidence of multiple paternity in a species and or population and primarily arises in species with no or low parental care, high mate-encounter rate, and cost of mating and because of strong mate selection (Lee et al., 2007; Uller & Olsson, 2008). Other population-specific characteristics, such as low genetic diversity and a high degree of inbreeding, are selective forces that are likely to trigger multiple paternity in reptiles (Lee et al., 2007; Uller & Olsson, 2008). In crocodylians, multiple paternity has been reported in *Crocodylus moreletti*, *Crocodylus latirostris*, *Alligator mississippiensis*, *Crocodylus porosus*, *Alligator sinensis*, and *Crocodylus crocodiles*. Multiple paternity in crocodylians is a well-known phenomenon occurring in up to 95% of instances, often with a contribution of up to ten males in a single clutch of eggs (Isberg, 2022; Oliveira et al., 2014).

An understanding of the mating system in the freshwater crocodile is essential in guiding the ongoing translocation. This will also help advance the captive breeding program management and conservation of the species. Nevertheless, due to the limited number of offspring analysed per clutch of the nest, the present study could not confirm each male's contribution to the nest. Further, many questions, such as (a) do diversity or density determines the incidence of multiple paternity and (b) do large males

contribute to higher proportionate and fertile clutches in freshwater crocodylians, remain unanswered. Further, the analysis could not be performed due to low polymorphism in nuclear microsatellites gharial population.

CHAPTER 5

SUMMARY

5.1 SUMMARY

Human-mediated loss of habitats through modification and fragmentation coupled with environmental change are causing population decline and range contraction in several wild species (Langridge et al., 2020; Weeks et al., 2011). In an attempt to halt and reverse the decline and improve the status of species – Conservation translocation, defined as the ‘deliberate movement of animals from one area to another, has become a strategy integral to the management of wild populations (Dodd & Seigel, 1991; IUCN/SSC, 2013; Wolf et al., 1996). In recent decades, conservation translocation has been increasingly recommended to improve population status and resilience in response to climate change (Berger-Tal et al., 2020; Griffith et al., 1989). Despite its importance and widespread recognition in achieving conservation goals and improving species resilience, the outcome of such a program often remains below par and variable due to the complex interplay between factors determining its success (Berger-Tal et al., 2020; Bubac et al., 2019). A range of factors has been deemed essential for the success of the translocation program, such as prior knowledge of the ecological role of focal species, availability of suitable habitat, climatic requirements, life-history traits, genetic diversity of the source population, and follow-up post-release monitoring (Bubac et al., 2019; IUCN/SSC, 2013; Morris et al., 2021; Weeks et al., 2015). While such programs often spend a tremendous effort on acquiring information on ecological and demographic factors, they inadvertently lack consideration of genetics for follow-up or post-release monitoring (Bubac et al., 2019).

The crocodylian populations in India experienced a severe population decline and range contraction until the mid-20th century, which led the authorities to forewarn the extinction risk of the crocodiles and proposed commercial regulation of the crocodile trade (Bustard, 1975; Hussain, 1999; Whitaker & Basu, 1982). The proposed action led to the promulgation of national and international wildlife regulations prohibiting the trade in crocodile skins and parts. The crocodile population in India had become so depleted that the authorities recognized that merely prohibiting trade would not be adequate to reverse the population decline and highlighted implementing active conservation strategies for crocodylian conservation in India. Subsequently, the crocodile conservation program “Project Crocodile” was initiated to restore these species in the wild. The crocodile populations responded quickly to the conservation efforts and began to increase in numbers (Nair et al., 2012; Singh, 1985).

In India, the crocodile conservation program began in the mid-1970s and has been one of India’s longest-running conservation initiatives. Since the start of the crocodile conservation program, research has been focused mainly on field ecology and biology. Several studies have been conducted on ecological and demographic features of gharial and mugger (Hussain, 2009; Hussain, 1999; Hussain, 1991; Hussain et al., 2013; Katdare et al., 2011; Lang et al., 2018; Nair et al., 2012; Sharma & Singh, 2015; Singh, 1978, 1985). Nonetheless, the genetic assessment status of these sympatric crocodylian species in these rivers is lacking (Jogayya et al., 2013). Hence, the study of the genetic status of these species is imperative, as past evaluation of the conservation program is solely based on the ecology and demographic of the species (Soorae, 2008).

The genetic assessment of the two sympatric crocodylian species revealed low to moderate levels of genetic diversity in these species. The genetic diversity observed at seven polymorphic nuclear microsatellite loci was low in the gharial population of Chambal River, while diversity at ten loci was moderate to high in the mugger population in Chambal. The genetic diversity of a species is influenced by its geographic range, abundance, demography, and life-history traits. Narrow-ranging, less abundant, and long-lived species with demographically challenged population history tend to have lower genetic diversity than widely distributed, abundant species with stable demographic history (Eckert *et al.*, 2008; Ellegren & Galtier, 2016; Romiguier *et al.*, 2014). The low abundance, narrow distribution range and complex life-history traits of the gharial may have resulted in the observed low level of genetic diversity. In contrast, the overall genetic diversity indices in mugger showed moderate values (Na-3.18; Ar-2.75; Ho-0.53; and He-0.48) relatively higher than gharial. The difference in diversity is perhaps due to the generalist nature of mugger, i.e., broader dietary choice and versatility in adapting to various habitats, which might have buffered the impact of habitat fragmentation, environmental fluctuations, and anthropogenic pressures. These adaptations have helped mugger thrive in diverse aquatic habitats, from ponds to rivers and in and around urban areas. The muggers are better at navigating terrestrial habitats than gharials. Due to their strong limbs, they can lift their bodies to walk – ‘high-walk’ and can thus move over great distances. This creates the possibility of mugger migrating from the tributaries and other water bodies to the more favourable habitat of the Chambal River. To reduce conflict, the local forest department often has to move mugger individuals from surrounding human

habitation. Such individuals released into the Chambal River in an unstructured manner potentially contribute to the increasing population as well as genetic diversity. The observed pattern of genetic diversity is in line with species ecology and generalist species retaining high diversity in contrast to specialist species living in similar habitats and environmental conditions. Nevertheless, the gharial population needs greater care and management interventions to reduce the negative consequences of low genetic diversity and maintain the genetic diversity for long-term persistence.

The presence of population genetic structure was inferred by Structure using sampling location as *prior* but not by multivariate analysis DAPC. The observed pattern could have multiple reasons: First, the use of a *prior* is helpful when the genetic differentiation between the location is extremely low, as observed in the case of gharial. Secondly, the Bayesian approach implemented in Structure is a model-based approach that largely depends on the assumption of population genetics models. In contrast, DAPC is a model-free approach that does not rely on assumptions population genetics models or makes bare basics assumptions. Hence, the result of DAPC, in this case, is more reliable and appropriate. The observed low level of differentiation and an admixed population structure suggested by Structure (*de novo* approach) and DAPC hint towards possible intermixing of gharial individuals during the translocation program when eggs from both the Chambal and Girwa Rivers were reared in the Kukrail center. Additionally, an exceptionally higher proportion of admix individuals suggests recent gene-flow between previously separated populations.

Furthermore, a relationship was observed between the two populations, alluding to migration between the two populations. However, due to the lack of any

apparent geographical linkage between the Chambal and Girwa rivers, the relation can be attributed to the human-mediated inter-mixing of individuals during the translocation program. It is important to note that the observed migration rate from Chambal to Girwa was higher than that from Girwa to Chambal, which could be due to a large number of eggs being sourced from Chambal and later released in the Girwa River.

Multiple paternity in crocodylians is a well-known phenomenon occurring in up to 95 % of instances, often with a contribution of up to five males in a single clutch of eggs. The paternity analysis provides multiple benefits, such as fertilization assurance, additional partners to care for hatchlings, and genetic benefits. Multiple paternity in species is viewed as an evolutionary or behavioral mechanism linked to increased reproductive success, genetic fitness, and social benefits. Extra-pair paternity (polyandry) increases the effective population size, genetic variation, and evolutionary potential of species. The present study confirms the incidence of multiple paternity in one of the mugger clutches assessed for multiple paternity. However, the incidence of multiple paternity in gharial could not be determined due to low statistical power (combined exclusion probability and probability of detecting multiple paternity) obtained using seven polymorphic nuclear microsatellites loci.

5.2 CONSERVATION IMPLICATION

The study of species' genetic status has become an essential component in managing and monitoring the recovery of species at risk. Nonetheless, despite a long history of conservation efforts, limited information is available on the genetic status of freshwater sympatric crocodylian species. The lack of information has hindered, if not

precluded, effective conservation planning of these species in the past and continues to be a blind spot for the conservation of crocodylians in India. To date, there is no information available on the genetic status of wild gharial and mugger populations. This study provides essential genetic information on both the crocodylians and enables understanding of species-specific traits, such as dispersal and mating systems, that are otherwise difficult to answer. This information is also vital for future translocations and research. The conservation implication based on the findings of the study are as follows:

- This study highlighted low levels of genetic diversity in the largest managed gharial populations of gharial in the wild, which are considerably lower than what has been previously reported in captive populations (Jogayya *et al.*, 2013). A low proportion of polymorphic markers and low polymorphism was observed in species-specific and cross-species microsatellite loci. Hence, it remains crucial to develop a suite of novel microsatellite loci for population genetic studies, and range-wide genetic assessment of the gharial population is necessary to substantiate the finding of this study and inform the conservation decisions.
- Gharial populations inhabit different biogeographic zones of India and are assumed to be adapted to specific environmental conditions, yet translocation programs have not considered the effects of genetic intermixing among populations. Such intermixing of populations without prior knowledge of the genetic structure can potentially threaten the genetic integrity and distinctiveness of the resident populations (IUCN/SSC, 2013).

- Moreover, the gharial is a highly conservation-dependent species, and translocations without assessing the genetic status of the source populations may further deteriorate the level of genetic diversity in the translocated populations. Hence, it is recommended to restrict the inter-population release and opt for intra-population release of individuals to prevent further intermixing of the gene pool until information on the genetic status of the extant wild and captive gharial populations using an extensive dataset of mitochondrial and microsatellite markers is available.
- Information on the genetic status will assist in the identification of potential source populations and maintain adequate levels of genetic diversity to secure the continuing persistence of gharials in the wild.
- Higher diversity in mugger, which is better at adaptation, may lead to increased competition with the gharial these rivers. This emphasizes the need for monitoring mugger as a gharial conspecific to effectively manage both the crocodiles in the same environment.

Freshwater species are becoming more and more susceptible as a result of anthropogenic stressors. The impacts are likely to become evident and widespread when coupled with global climate change. Climate change can have a significant impact on both the environment and the species that inhabit it. Changes in temperature can alter the timing of important biological events such as migration and breeding, disrupting the food chain and reducing food availability. Crocodylians are highly dependent on temperature regimes to regulate their body temperature and control their reproductive cycles. Also, declining river water levels can result in a reduction of

suitable habitats for these species. These factors can interact in complex ways to create a challenging environment for many species, leading to declines in population size, changes in distribution, and in some cases. In such conditions, species with low adaptive potential, often measured through the level of genetic diversity, may become highly vulnerable to extinction.

Information on the genetic status of two threatened freshwater crocodylians will aid in planning their conservation and management strategies. The finding of the study is vital for assessing and evaluating the recovery of the species in terms of genetic recovery, essential for guiding the ongoing reinforcement program of the species.

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APPENDICES

APPENDIX 1: DNA EXTRACTION PROTOCOL-PHENOL CHLOROFORM (SAMBROOK ET AL. 1989)

1. Chop the sample using a sterile knife/blade (**80-100mg**) and keep it in an Eppendorf tube.
2. Add **600µl** lysis buffer + **60µl** of 20 % SDS + **25µl** proteinase K.
3. Keep at **56°C** in a shaking water bath for **24-48 hours**.
Centrifuge at **10000rpm** for **10mins**.
4. Collect the **supernatant** in a new sterile Eppendorf tube.
5. Add an **equal amount** of **saturated phenol** to it and mix well for **10mins** using hand.
Centrifuge at **10000rpm** for **10mins**.
6. Collect the **supernatant** in a new sterile Eppendorf tube.
7. Add **300µl** of chloroform: Isoamyl alcohol (24:1) + **300µl** of saturated phenol.
Centrifuge at **10000rpm** for **10mins**.
8. Collect the **supernatant** in a new sterile Eppendorf tube.
9. Add **600µl** of chloroform: Isoamyl alcohol (24:1) and mix well for **10mins**.
Centrifuge at **10000rpm** for **10mins**.
10. Collect the **supernatant** *carefully* in a new sterile Eppendorf tube and add **0.1(10%) volume** of 3M Sodium acetate (pH 5.2).
11. Mix well and leave it for **10-15minutes** at room temperature and add **0.6 (60%) volume** of chilled isopropyl alcohol.
Centrifuge at **10000rpm** for **10mins**.
12. *Discard* the supernatant *carefully* and add **500µl** of **70% ethanol**.
Centrifuge at **10000rpm** for **10mins**.

13. *Discard* the supernatant *carefully* and add **500µl** of absolute ethanol.

Mix gently and Centrifuge at **10000rpm** for **10mins**.

14. *Discard* the supernatant *carefully* and keep the Eppendorf tube on the block heater for **10mins** to dry the pellet

15. *Dissolve* the DNA in AE buffer and keep it on a block heater to dissolve the DNA.

APPENDIX 2: POLYMERASE CHAIN REACTION- MIX PREPARATION FOR AMPLIFICATION OF MITOCHONDRIAL CONTROL REGION FRAGMENT

Components	Per reaction (μL)
10x DreamTaq buffer	2.0
0.2 mM of each dNTP	2.0
Primer (forward)	0.25
Primer (reverse)	0.25
DreamTaq DNA polymerase	0.1 (0.5 units per reaction)
Nuclease free water	13.4
DNA template	2.0
Total volume	20.0

APPENDIX 3: DNA SEQUENCING - MIX PREPARATION FOR AMPLIFICATION OF MITOCHONDRIAL CONTROL REGION FRAGMENT

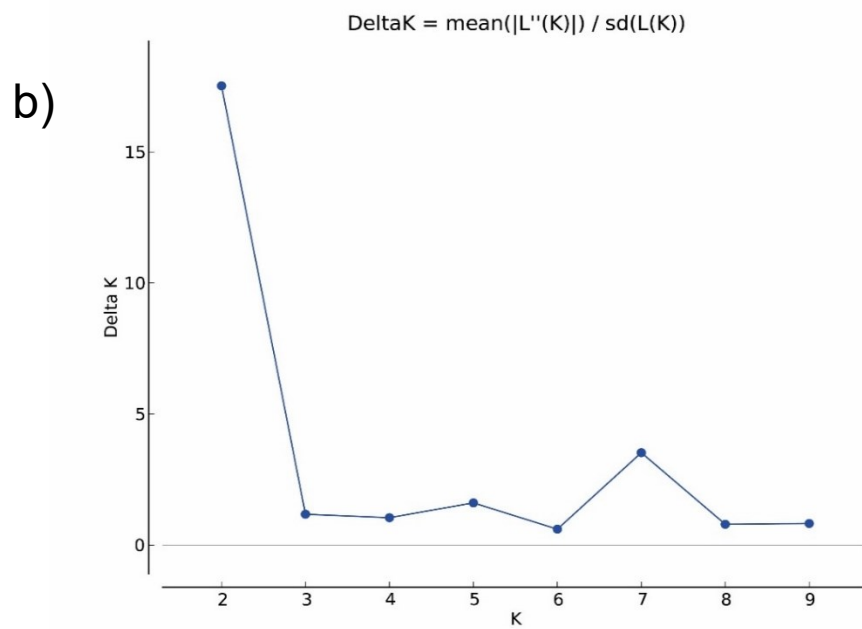
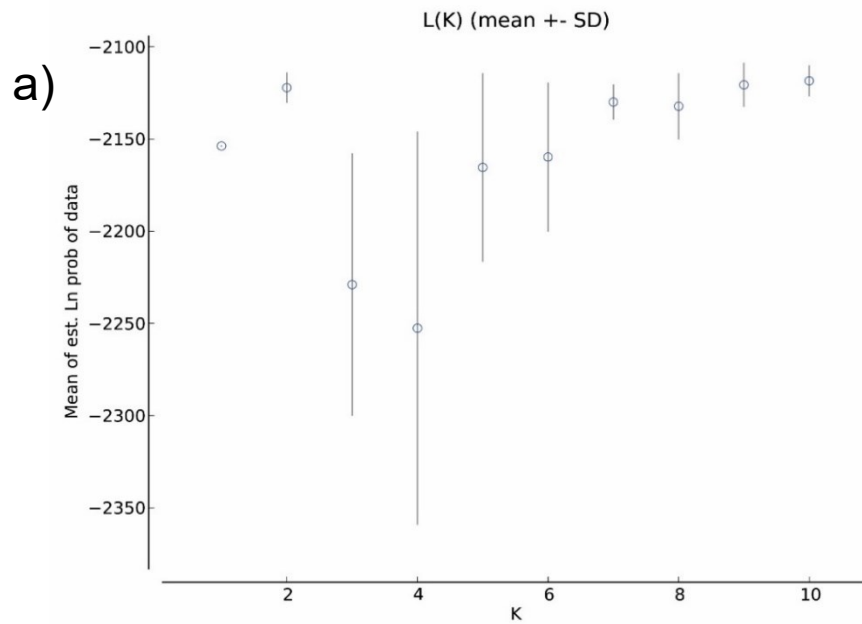
Components	Per reaction (μL)
Big dye v 3.1 ready reaction mix	0.25
5x sequencing buffer	2.0
Primer	0.2
PCR product (amplicon)	1.0
Nuclease free water	6.55
Total volume	10.00

**APPENDIX 4: DETAIL LIST OF THE 42 NUCLEAR MICROSATELLITES LOCI SCREENED IN
THE PRESENT STUDY**

Locus name	Repeat	Species	Reference
4HDZ27	-di-	<i>Crocodylus mindorensis</i>	Hinlo et al., 2014
4HDZ35	-di-	<i>Crocodylus mindorensis</i>	Hinlo et al., 2014
Amiu-102	-di-	<i>Alligator mississippiensis</i>	Glenn et al., 1998
Amiu-202	-di-	<i>Alligator mississippiensis</i>	Davis et al., 2002
C391	-di-	<i>Crocodylus acutus</i>	FitzSimmons et al., 2000
Cc_D07	-di-	<i>Caiman crocodilus</i>	Oliveira et al., 2010
Cc_E08	-di-	<i>Caiman crocodilus</i>	Oliveira et al., 2010
Cj119	-di-	<i>Crocodylus johnstoni</i>	FitzSimmons et al., 2001
Cj16	-di-	<i>Crocodylus johnstoni</i>	FitzSimmons et al., 2000
CpDi04	-di-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP107	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP121	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP1306	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP1401	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP203	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP208	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP309	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP3220	-tetra-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpP722	-tetra-di-	<i>Crocodylus porosus</i>	Miles et al., 2008
CpSSR01_F	-di-	<i>Crocodylus palustris</i>	Aggarwal et al., 2014
CpSSR03_F	-di-di-	<i>Crocodylus palustris</i>	Aggarwal et al., 2014
CpSSR04_F	-di-	<i>Crocodylus palustris</i>	Aggarwal et al., 2014
CpSSR12	-di-	<i>Crocodylus palustris</i>	Aggarwal et al., 2014
CpSSR13	-di-	<i>Crocodylus palustris</i>	Aggarwal et al., 2014
CpSSR14	-di-	<i>Crocodylus palustris</i>	Aggarwal et al., 2014
CpSSR19_F	-di-	<i>Crocodylus palustris</i>	Aggarwal et al., 2014
CUD68	-di-	<i>Crocodylus acutus</i>	FitzSimmons et al., 2000
Cy-I14	-di-	<i>Caiman yacare</i>	Godshalk et al., 2006
G13_1	-tri-di-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
G13_11	-tetra-di-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
	-tetra-di-		Jogayya et al., 2013
G13_14	tetra-	<i>Gavialis gangeticus</i>	
G13_15	-tetra-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
G13_16	-tetra-di-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013

G13_18	-di-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
G13_2	-tetra-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
G13_5	-tetra-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
G13_6	-tetra-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
G13_7	-tetra-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
G13_8	-tetra-	<i>Gavialis gangeticus</i>	Jogayya et al., 2013
	-di-tetra-		Jogayya et al., 2013
G13_9	di-	<i>Gavialis gangeticus</i>	
TGE2	-di-	<i>Alligator sinensis</i>	Yu et al., 2011
TM23	tetra-	<i>Tomistoma schigelli</i>	Chang et al., 2004

APPENDIX 5: THE PLOT OF A) THE HIGHEST MEAN LIKELIHOOD $L(K)$ AND B) ΔK USING SAMPLING LOCATION AS A PRIORI





OPEN

Microsatellite analysis reveals low genetic diversity in managed populations of the critically endangered gharial (*Gavialis gangeticus*) in India

Surya Prasad Sharma¹, Mirza Ghazanfarullah Ghazi¹, Suyash Katdare¹, Niladri Dasgupta¹, Samrat Mondol¹, Sandeep Kumar Gupta¹ & Syed Ainul Hussain¹✉

The gharial (*Gavialis gangeticus*) is a critically endangered crocodylian, endemic to the Indian subcontinent. The species has experienced severe population decline during the twentieth century owing to habitat loss, poaching, and mortalities in passive fishing. Its extant populations have largely recovered through translocation programmes initiated in 1975. Understanding the genetic status of these populations is crucial for evaluating the effectiveness of the ongoing conservation efforts. This study assessed the genetic diversity, population structure, and evidence of genetic bottlenecks of the two managed populations inhabiting the Chambal and Girwa Rivers, which hold nearly 80% of the global gharial populations. We used seven polymorphic nuclear microsatellite loci and a 520 bp partial fragment of the mitochondrial control region (CR). The overall mean allelic richness (A_r) was 2.80 ± 0.40 , and the observed (H_o) and expected (H_e) heterozygosities were 0.40 ± 0.05 and 0.39 ± 0.05 , respectively. We observed low levels of genetic differentiation between populations ($F_{ST} = 0.039$, $P < 0.05$; $G'_{ST} = 0.058$, $P < 0.05$ Jost's $D = 0.016$, $P < 0.05$). The bottleneck analysis using the M ratio (Chambal = 0.31 ± 0.06 ; Girwa = 0.41 ± 0.12) suggested the presence of a genetic bottleneck in both populations. The mitochondrial CR also showed a low level of variation, with two haplotypes observed in the Girwa population. This study highlights the low level of genetic diversity in the two largest managed gharial populations in the wild. Hence, it is recommended to assess the genetic status of extant wild and captive gharial populations for planning future translocation programmes to ensure long-term survival in the wild.

The order Crocodylia is represented by 27 species and three families: Alligatoridae, Crocodylidae and Gavialidae¹. India harbours three crocodylian species: the gharial (*Gavialis gangeticus*), the mugger or marsh crocodile (*Crocodylus palustris*), and the saltwater crocodile (*Crocodylus porosus*). The gharial has the narrowest distribution range and is the most threatened species among all other crocodylian species occurring in the Indian subcontinent. Until the early twentieth century, the gharial was widely distributed in the Indus, Ganges, Mahanadi, Brahmaputra, Kaladan and Irrawaddy River systems spanning across Pakistan, India, Nepal, Bangladesh, Bhutan, and Myanmar^{2–4}. Over the years, the gharial has suffered a population decline of over 80% and substantial range contraction due to habitat loss, poaching, and mortalities in passive fishing^{5–7}. The species is now extinct in Myanmar, Bhutan and Pakistan. The extant gharial population is restricted to a few major river systems in India, Nepal and Bangladesh. It is presently listed as 'critically endangered' on the IUCN Red List of threatened species and is highly conservation dependent⁷.

In India, by the early 1970s, the gharial was restricted to few isolated locations in the Ganga, Mahanadi and Brahmaputra River systems². Due to the alarming decline in the gharial populations throughout its range, the Government of India listed it in Schedule I of the Indian Wild Life (Protection) Act, 1972 to provide enhanced protection. Subsequently, conservation translocation programmes, including reintroduction and restocking, were initiated in 1975 to restore its population in the wild⁵. As a part of these programmes, eggs collected from

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the wild were hatched and reared in rehabilitation centres, and individuals after attaining a length of 1.2 m were translocated into suitable habitats within the newly created protected areas^{5,6}.

In India, the head-start gharial conservation programme was initiated at the Chambal, Girwa and Mahanadi Rivers, where adult breeding populations existed. Initially, four facilities were established, viz. (1) Gharial Rehabilitation Centre, Tikarpada; (2) Gharial Rehabilitation Centre, Katarniaghat; (3) Gharial Rehabilitation Centre, Kukrail; and (4) Deori Gharial Rearing Centre, Morena targeting the Mahanadi, Girwa and Chambal populations. Hatchlings reared at these centres were released at the targeted rivers. In the Kukrail Centre, gharial hatchlings from 240 eggs collected from Chambal and 38 eggs collected from Girwa were reared during 1975^{8,9}. Although there is no record, the hatchlings from these two rivers were likely intermixed while rearing. The hatchlings produced in Kukrail and Deori were also translocated to several other rivers, viz. Son, Ken and Ramganga, some of which have started breeding¹⁰. Eggs were also collected from the Narayani River in Nepal, and hatchlings were released in the Girwa river. Additionally, conservation breeding was initiated in several zoos, such as the Nandankanan Biological Park and Madras Crocodile Bank, which contributed stocks for translocation¹¹. A male gharial from the Frankfurt Zoo, Germany, was obtained, which also contributed to developing stocks in Odisha for translocation¹². All translocation sites are now protected as wildlife sanctuaries under the Indian Wild Life (Protection) Act, 1972. In the last four decades, over 5000 gharial individuals have been translocated in more than 12 rivers in India, with over 3500 individuals in the Chambal and Girwa Rivers¹². At the beginning of the restocking programme, the gharial population in the Chambal River was 107 individuals, and now, it is approximately 1675 individuals of different ages and sexes^{4,13}. The Chambal River within the National Chambal Sanctuary (425 km) has been a stronghold of the gharial population since the initiation of the translocation programme. The Girwa River originally had a small population of approximately 34 individuals of different ages and sexes¹¹ and now has 43 adult individuals restricted to a 20 km stretch of the Girwa River falling within the Katarniaghat Wildlife Sanctuary. Presently, these two populations hold nearly 80% of the extant wild populations of gharial⁷.

Effective translocation programmes of endangered species essentially depend on prior knowledge of the ecological role, availability of suitable habitat, climatic requirements, behaviour, life-history traits, adequate post-release monitoring, demographic, behavioural and ecological aspects^{14–16}. The effectiveness of any translocation programme is also influenced by the level of genetic diversity of the source population¹⁷, affecting individual and population fitness, resilience against environmental change and long-term persistence¹⁸. However, most translocation programmes rarely evaluate this factor¹⁷. Therefore, understanding the genetic status of the translocated population is vital for determining the effectiveness of translocation. Since the initiation of the gharial conservation programme, several studies have been carried out to determine their ecological features^{2,4,6,13,19–21}. Nevertheless, studies on the genetic status of wild and captive gharial populations are limited^{22,23}.

In this study, we aim to assess the genetic diversity, population genetic structure and evidence of genetic bottlenecks using nuclear microsatellite loci and the mitochondrial control region (CR) of the two largest managed populations of gharials inhabiting the Chambal and Girwa Rivers (Fig. 1) to answer following research questions: (a) Do the two largest managed gharial populations differ in terms of genetic diversity? (b) Do the Chambal and Girwa River populations have their own genetic signatures? (c) If so, what is the level of differentiation? and (d) Did the demographic decline suffered by gharial populations in the last century also induce a genetic bottleneck?

Results

Microsatellite selection, screening and genotyping. Out of the 27 nuclear microsatellite loci (11 species-specific and 16 cross-species) screened, 18 loci showed successful amplification, and nine loci failed to amplify using known gharial samples (see Supplementary Table S1 online). We successfully genotyped 18 loci for 348 samples (Chambal = 232; Girwa = 116). Furthermore, out of 18 loci, 11 loci were found to be monomorphic and thus removed from further analyses. Overall, we used microsatellite genotype data of seven polymorphic loci from 348 gharial samples for population genetics analyses.

Genotyping quality and error rates. The polymorphic loci average amplification success rate was 96.09% for Chambal samples and 95.38% for Girwa samples. The quality index across the seven polymorphic loci was 0.86 ± 0.01 (mean $\pm$ SE) in Chambal and 0.89 ± 0.01 in Girwa (see Supplementary Figs. S1 and S2 online). The average allelic dropout (ADO), false allele (FA) and null allele frequency across polymorphic loci were below 5% in both populations (see Supplementary Table S2 online). We could not detect the occurrence of large allele dropout in our data.

Genetic variation. The cumulative probability of identity (PID biased) value of the panel of seven polymorphic markers was 1.74×10^{-3} , and the probability of identity (PID sibs) was 4.84×10^{-2} . We identified a total of 228 (Chambal = 162; Girwa = 66) distinct individuals using multilocus genotype data. The genetic diversity estimates for the Chambal and Girwa populations are summarised in Table 1. The number of alleles observed at each locus ranged from 2 to 7. The overall mean allelic richness was 2.80 ± 0.40 , and the observed (H_o) and expected (H_e) heterozygosities were 0.40 ± 0.05 and 0.39 ± 0.05 across seven polymorphic loci, respectively. The mean allelic richness estimated using the rarefaction approach was 2.91 ± 0.60 in the Chambal population and 2.48 ± 0.48 in the Girwa population. The mean observed and expected heterozygosities were 0.42 ± 0.07 and 0.41 ± 0.06 in the Chambal and 0.42 ± 0.08 and 0.42 ± 0.09 in the Girwa populations, respectively. The inbreeding coefficient (F) was -0.03 for both populations. None of the loci showed significant deviation from HWE following Bonferroni correction.

Population genetic structure, differentiation and migration. The Bayesian approach implemented in Structure v2.3.4 identified two ($K=2$) optimum number of clusters inferred by the likelihood distribution

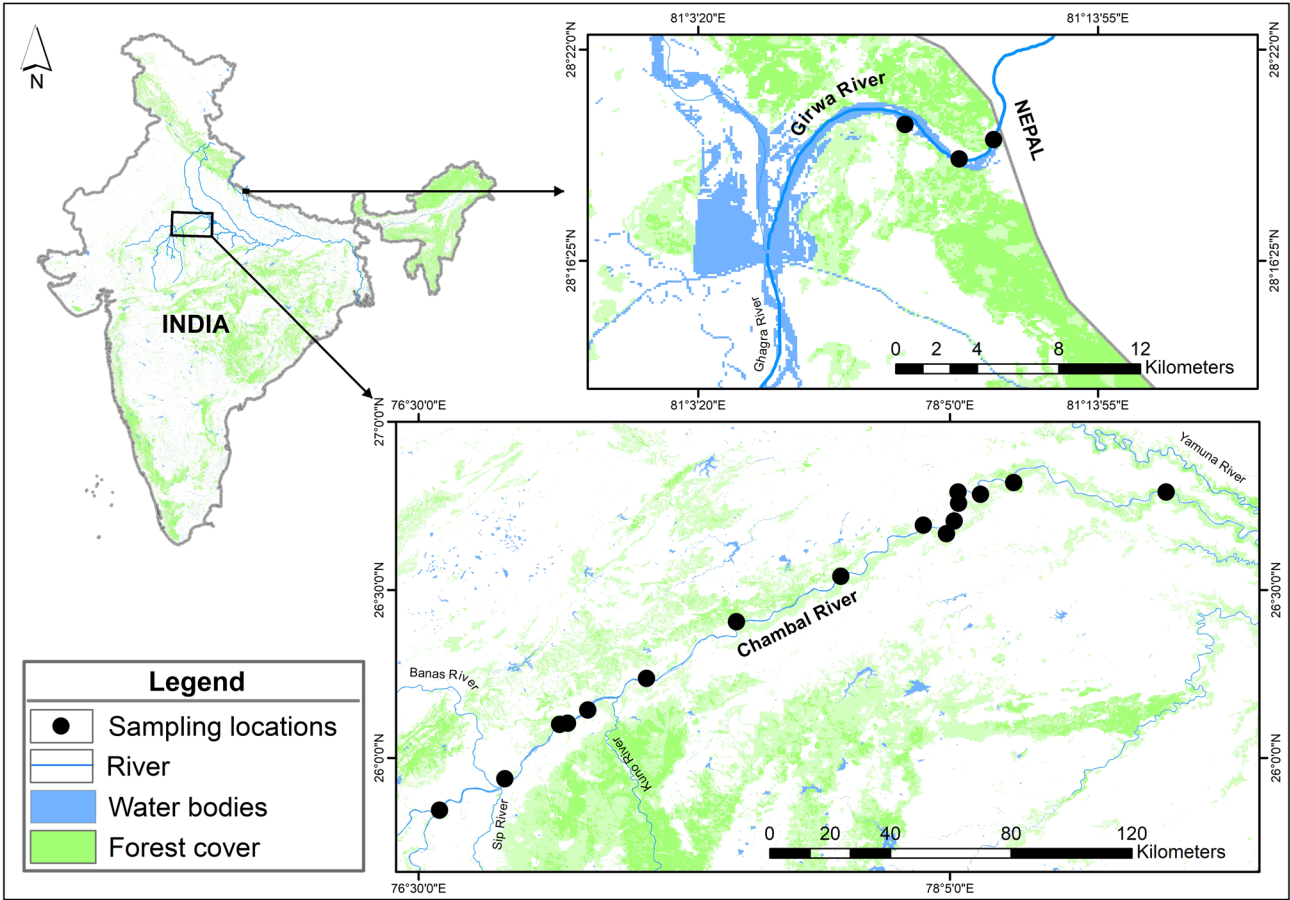


Figure 1. Map showing detailed representation of the study area and sampling locations along the Chambal and Girwa Rivers. The map was prepared using ArcGIS v.10.3.1 software developed by ESRI (<https://www.esri.com>).

Population	Chambal river						Girwa river					
Locus	N	Na	Ar	Ho	He	F	N	Na	Ar	Ho	He	F
G13_7	160	2	2.0	0.31	0.33	0.07	66	2	2.0	0.50	0.49	−0.02
Cj16	158	2	2.0	0.27	0.27	−0.01	66	2	2.0	0.03	0.03	−0.02
G13_2	162	2	2.0	0.54	0.50	−0.09	66	2	2.0	0.56	0.48	−0.17
G13_5	153	3	3.0	0.24	0.23	−0.05	Monomorphic					
G13_8	159	7	6.3	0.74	0.67	−0.11	66	5	5.0	0.53	0.63	0.16
G13_14	160	3	3.0	0.42	0.42	0.00	66	3	3.0	0.42	0.52	0.19
G13_16	160	2	2.0	0.44	0.44	−0.01	66	2	2.0	0.47	0.36	−0.31
Mean		3	2.91	0.42	0.41	−0.03		2.67	2.48	0.42	0.42	−0.03
SE		0.69	0.60	0.07	0.06	0.02		0.49	0.48	0.08	0.09	0.08

Table 1. Genetic diversity of 228 individuals at seven microsatellite nuclear loci. Number of individuals sampled (N), number of alleles per locus (Na), standardised number of alleles (Ar), observed heterozygosity (Ho), expected heterozygosity (He), Inbreeding coefficient (F).

$L(K)$ and ΔK estimates (see Supplementary Fig. S3 online). All Girwa samples were assigned to cluster-I, with an average proportion of membership (q) of 0.97, and Chambal samples were assigned to cluster-II, with $q = 0.82$ (Fig. 2a). Genetic differentiation measures were low but significant as derived using both the fixation indices: $F_{ST} = 0.039$ ($P < 0.05$); $G'_{ST} = 0.058$ ($P < 0.05$) and allelic differentiation index Jost's $D = 0.016$ ($P < 0.05$).

The discriminate analysis of principal component (DAPC) identified 20 genetic clusters associated with the lowest Bayesian information criterion (BIC) (see Supplementary Fig. S4 online). All the identified clusters ($K = 20$) showed an overlapping pattern with no clear structuring (Fig. 2b). Furthermore, the recent migration rate (m) was 0.12 (95% CI 0–0.26) from Chambal to Girwa River and 0.02 (95% CI 0–0.05) from Girwa to Chambal.

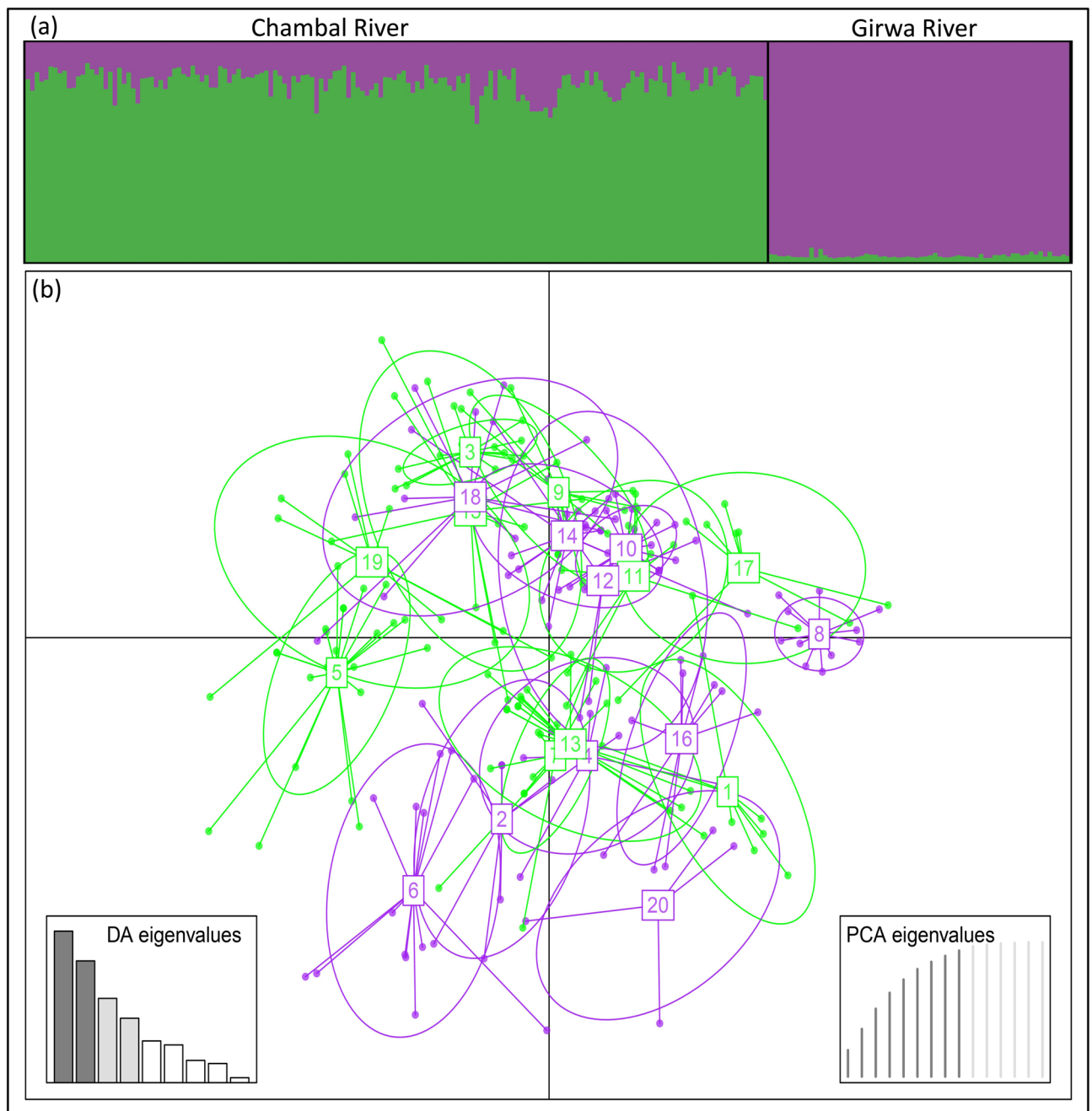


Figure 2. (a) Population genetic structure of 228 gharial individuals derived using a Bayesian clustering approach implemented in STRUCTURE. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster. (b) Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 228 gharial individuals. This scatterplot shows the first two principal components of the DAPC.

Bottleneck. The M ratio calculated across polymorphic loci was 0.31 ± 0.06 in Chambal and 0.41 ± 0.12 in Girwa, indicating a genetic bottleneck in both populations. The heterozygosity excess test (HET) using the stepwise mutation model (SMM) (Chambal $P=0.28$; Girwa $P=0.08$) and the two-phase model (TPM) (Chambal $P=0.18$; Girwa $P=0.08$) failed to detect the signature of a genetic bottleneck. We also obtained a normal L-shaped allelic distribution for both populations.

Mitochondrial DNA variation. We observed two mitochondrial CR haplotypes H1 in 58 individuals (92%) and H2 in five individuals (8%) with a single parsimony-informative site (at the 375-nucleotide position) in the Girwa population. Haplotype, H1 was shared by both the Girwa and Chambal populations²², whereas H2 was unique to the Girwa population. The haplotype diversity was (mean $\pm$ SD) 0.148 ± 0.057 , and nucleotide diversity was 0.00029 ± 0.00011 in the Girwa population.

Discussion

This study is the first comprehensive genetic assessment of the critically endangered gharial and provides crucial baseline information essential for guiding the ongoing conservation efforts. The genetic diversity observed at seven polymorphic nuclear microsatellite loci was low in the gharial populations of Chambal and Girwa. Both populations had similar mean heterozygosities, and the allelic richness (Ar) was marginally higher in the Chambal population (Table 1). The overall heterozygosities at nuclear loci in the wild ($H_o = 0.40 \pm 0.05$; $H_e = 0.39 \pm 0.05$) were lower than the previously reported estimates in captive ($H_o = 0.92 \pm 0.02$; $H_e = 0.65 \pm 0.02$) gharial populations using the same set of microsatellite loci. The estimated mean allelic richness was also lower in the wild population (Chambal = 2.91 ± 0.60 ; Girwa = 2.48 ± 0.48) than that reported in captivity (5.5 ± 0.5)²³. A low level of heterozygosity is not rare in wild crocodylians. Similar levels of heterozygosity have been reported in other crocodylians, including *Alligator sinensis*²⁴, *Alligator mississippiensis*²⁵, *Caiman yacare*²⁶, *Crocodylus siamensis*²⁷, *Crocodylus mindorensis*²⁸, *Crocodylus moreletii*^{29,30}, and *Crocodylus palustris*^{31,32}, as well as in other vertebrate species that have experienced demographic bottlenecks³³. Moreover, the Girwa population exhibits two and Chambal population exhibits a single mitochondrial CR haplotype²². The presence of extremely low variation at a hypervariable locus is surprising because, in other crocodylian species, the reported haplotypic diversity at mitochondrial CR is considerably higher^{34–38}. The genetic diversity of a species is influenced by its geographic range, abundance, demography, and life-history traits. Narrow-ranging, less abundant and long-lived species with demographically challenged population history tend to have lower genetic diversity than widely distributed, abundant species with stable demographic history^{39–41}. The low abundance, narrow distribution range, complex life-history traits, and unstable demography of the gharial may have resulted in the observed low level of genetic diversity.

The coefficient of inbreeding (F), primarily measures the deviation from HWE (where $F = 0$ indicates that the population is at HWE) and is often only weakly correlated with inbreeding and fitness^{42,43}. The estimate is likely to be affected by several factors including use of a limited number of loci, closely related individuals, genetic bottleneck, gene flow and admixture^{44–46}. Additionally, populations that experience demographic bottlenecks do not necessarily become inbred. Our estimate of $F \approx 0$, shows that the populations are at or near HWE.

Population genetic structure, differentiation, and migration. The presence of population genetic structure was supported by Structure (Fig. 2a) and genetic differentiation indices ($F_{ST} = 0.039$, $P < 0.05$; $G'_{ST} = 0.058$, $P < 0.05$ Jost's $D = 0.016$, $P < 0.05$) but not by multivariate analysis DAPC (Fig. 2b). The Bayesian approach implemented in Structure is a model-based approach that largely depends on the assumption of population genetics models. In contrast, DAPC is a model-free approach that does not rely on population genetics models. DAPC performs better than Structure in characterizing population clusters⁴⁷. Therefore, we believe that the result of DAPC is more reliable and appropriate for our dataset. The observed low level of differentiation and an admixed population structure suggested by DAPC hint towards possible intermixing of gharial individuals during the translocation programme when eggs from both the Chambal and Girwa Rivers were reared in the Kukrail centre.

Furthermore, we observed a migratory relation between the two populations. This supports our hypothesis of possible mixing of individuals during the translocation programme. It is important to note that the observed migration rate from Chambal to Girwa was higher than that from Girwa to Chambal, which is possible due to large number of eggs were sourced from Chambal and later released in the Girwa River. We could not perform genetic structure, differentiation, and migration analyses using mitochondrial CR because of low variability in the analysed region.

Bottleneck. The estimates of the M ratio (Chambal = 0.31 ± 0.06 ; Girwa = 0.41 ± 0.12) were lower than the critical value ($M_C = 0.68$), confirming the signature of genetic bottlenecks in both populations. In contrast, the HET failed to detect the signature of a genetic bottleneck. This discrepancy between the results could be due to the limited statistical power of the two analyses. The M ratio can effectively detect genetic bottlenecks for a greater amount of time (up to 50 generations), whereas HET can efficiently detect bottleneck that occurred within a short time, $0.2–4.0 N_e$ (effective population size) generations^{48,49}. The gharial population has suffered two instances of range-wide population decline during the early-1970s and mid-1990s, when the population dwindled to less than 200 adult individuals in the wild^{6,20}. The average generation time estimates for gharial individuals is 25 years⁷, suggested that the latter decline in population occurred very recently (approx. 1–1.5 generation ago). Hence, the undetectability of the bottleneck through HET could be due to the recent bottleneck event. Additionally, bottleneck detection can be masked due to various confounding factors including time of occurrence, duration, magnitude, gene flow, pre-bottleneck genetic variability, mating system, sample size and a limited number of loci^{50–52}. Hence, there is a need to investigate both of these populations with extensive microsatellite loci to achieve high statistical power^{49,53,54}.

In conclusion, low polymorphism was observed in species-specific and cross-species microsatellite loci; it remains crucial to develop novel microsatellite loci for population genetic studies. We acknowledge that our study might have been influenced by the inclusion of closely related individuals from each nesting site. However, the sampling was conducted from a large number of nests spread across 19 nesting sites. Therefore, we believe that our sampling strategy potentially represents the level of genetic diversity of the studied populations and minimises the bias arising from closely related individuals.

Conservation implication. Gharial translocation is considered as one of the most successful species recovery programmes in the world¹² in terms of increasing the abundance of the species in the wild. However, despite a long history of conservation efforts, limited information on the genetic status of the gharial has hindered, if

not precluded, effective conservation planning. Gharial populations inhabit different biogeographic zones of India and are adapted to specific environmental conditions, yet translocation programmes have not considered the effects of genetic intermixing among populations. Such intermixing of populations without prior knowledge of the genetic structure can potentially threaten the genetic integrity and distinctiveness of the resident populations¹⁴. Our study augments the existing knowledge on the genetic status of the critically endangered gharial, which is vital for future translocations and research. This study highlighted low levels of genetic diversity and admixed structure in the two largest managed populations of gharial in the wild, which are considerably lower than the previously reported estimates of captive populations²³.

Moreover, the gharial is a highly conservation-dependent species, and translocations without assessing the genetic status of the source populations may further deteriorate the level of genetic diversity in the translocated populations. Hence, we recommend limiting the interpopulation release of individuals to prevent further intermixing of the gene pool until information on the genetic status of the extant wild and captive gharial populations using an extensive dataset of mitochondrial and microsatellite markers is available. Information on the genetic status will assist in the identification of potential source populations and maintain adequate levels of genetic diversity to secure the continuing persistence of gharials in the wild.

Methods

Research permits and ethical considerations. The Forest Department of Madhya Pradesh (Letter No. SL/Tech-1/8200), Rajasthan (Letter No. 1399) and Uttar Pradesh (Letter No. 3093 and 3778(A)) provided all required permissions for the survey and collection of the biological samples. As all samples were collected from either dead remains of hatchlings or hatched eggshells, no animal ethical clearance was required for this study.

Sample collection and DNA extraction. Sampling of a large number of adult individuals required for population genetic assessments has its own ethical and logistical constraints, especially when the species in question is critically endangered. Considering these restrictions, we collected tissue from the remains of dead hatchlings and chorioallantoic membranes from hatched eggshells shortly after hatching. Samples were collected from 16 nesting sites along the Chambal River and three nesting sites along the Girwa River during 2017 and 2018, respectively (Fig. 1). We collected 348 samples (Chambal = 232 samples and Girwa = 116 samples) for genetic assessment (see Supplementary Table S3 online). Out of 348 samples, 49 samples were unique (one sample per clutch), 138 samples were from a sibling (more than one sample per clutch), and 161 samples had no clutch information (see Supplementary Table S4 online). All samples were stored in absolute ethanol at room temperature and later at -20°C in the laboratory for long-term storage. Total genomic DNA was extracted from tissue (64 ± 19 mg) (mean $\pm$ SD) and chorioallantoic membrane using the phenol–chloroform method⁵⁵. Cotton swabs were used when chorioallantoic membrane was unable to separate from eggshells.

Microsatellite selection, screening and genotyping. We initially screened 11 of the 18 gharial specific microsatellite loci described by Jogayya et al.²³. However, only six of the eleven loci were found to be polymorphic. Hence, we conducted an exhaustive literature survey to identify the potential cross-species loci used across families Alligatoridae, Crocodylidae and Gavialidae. We found a total of 424 previously described microsatellite loci and listed the loci based on successful cross-species transferability, allele diversity, heterozygosity, polymorphic information content and allele range. Finally, we selected 16 cross-species loci developed in eight different crocodylian species for screening. Out of 16, a total of 12 loci were selected based on successful cross-species transferability in other crocodylian species. The remaining four loci were selected based on a number of alleles ≥ 4 , H_o and $H_e \geq 5$, $PIC \geq 0.5$ and allelic range < 300 bp. The list of microsatellite loci screened in the current study is provided in Supplementary Table S1 online.

Polymerase chain reactions (PCR) were performed in 10 μL reaction volumes containing 5 μL of 2 $\times$ QIAGEN Multiplex PCR master mix, 2 μL of 5 $\times$ Q-solution (QIAGEN Inc., Germany), and the labeled forward primer at 0.15 μM and the reverse primer at 0.15 μM and 2 μL of DNA template. The thermal profiles included an initial denaturation at 95°C for 15 min, followed by 35 cycles at 94°C for 40 s, T_a at 56 – 62°C (see Supplementary Table S1 online) for 60 s, 72°C for 60 s and a final extension of 72°C for 30 min. The amplified products were genotyped using the GeneScan 500 LIZ dye size standard (Applied Biosystems) in 3500XL Genetic Analyzer (Applied Biosystems). The alleles were scored using the program GeneMarker v2.7.4 (SoftGenetics, LLC) with combined automated allele scoring and validated through visual inspection. Three replicates of each sample were carried out to obtain reliable multilocus genotypes using noninvasive samples following a multiple-tube approach⁵⁶.

Mitochondrial CR DNA sequencing. We selected a 520 bp partial fragment mitochondrial CR to assess the genetic variation in gharials. We used primers (L15637 5'-GCATAACACTGAAAATGTTAAYATGG-3' and H16258 5'-CTAAAATTACAGAAAAGCCGACCC-3') described by Oaks (2011) to amplify the selected fragment⁵⁷. The PCRs were carried out in 20 μL volumes containing 2 μL of the DNA template, 10 μL of 2 $\times$ PCR buffer, 0.2 mM of dNTPs, 0.25 μL of each primer, and 0.1 μL (0.5 units) of DreamTaq DNA polymerase (Thermo Scientific). The thermal profile was 95°C for 5 min, followed by 35 cycles at 95°C for 35 s, 56°C for 40 s, 72°C for 45 s, and a final extension of 72°C for 10 min. The amplified products were visualised using a 2% agarose gel. The positive amplicons were cleaned up with Exonuclease-I (Thermo Scientific) and Shrimp Alkaline Phosphatase (Applied Biosystems), and sequenced using forward primers in 3500XL Genetic Analyzer (Applied Biosystems).

Data analysis. Genotyping quality and error rates. We obtained a consensus genotype and estimated the quality index for each locus genotyped following Miquel et al.⁵⁶. We scored each repeat with '1' if the genotype

was identical to the consensus and '0' if the genotype did not match the consensus due to any errors such as no amplification, allelic dropout, or false allele⁵⁶. We estimated the average amplification success as a percent of positive PCR amplification. We estimated the frequency of genotyping errors due to allelic dropout (ADO) and false allele (FA) following Broquet et al.⁵⁸. The frequency of null alleles was estimated using FreeNa⁵⁹ and occurrences of large allelic dropout using Micro-Checker v2.2.3⁶⁰.

We used samples with a quality index above 0.67 per locus (identical genotypes in two replicates out of three replicates) and a mean quality index across loci above 0.75 for analyses.

Genetic variation. We calculated the probability of identity PID (biased) and PID (sibs) using Gimlet v1.3.3⁶¹. We estimated summary statistics (number of alleles per locus, observed heterozygosity, expected heterozygosity, and inbreeding coefficient) using GenAlEx v6.0⁶² and deviations from Hardy–Weinberg equilibrium (HWE) for each locus using Bonferroni correction in Cervus v3.0.7⁶³. We also estimated allelic richness using a rarefaction approach implemented in HP-Rare v1.1 to account for the uneven sample size of the two populations⁶⁴.

Population structure, differentiation, and migration. We have estimated the genetic differentiation using fixation indices F_{ST} ⁶⁵, G'_{ST} ⁶⁶ and allelic differentiation index Jost's D' ⁶⁷ using the *strataG* package⁶⁸ implemented in R studio. The estimates were calculated using 10³ bootstrap iterations. We inferred the population genetic structure using the Bayesian approach implemented in Structure v2.3.4⁶⁹. Structure is a systematic model-based Bayesian clustering approach that uses allele frequencies at each locus to infer the population structure. The analysis was performed for 1–10 clusters (K). For each K , ten iterations were run under the admixture model with correlated allele frequencies and sampling location as a priori. The LOCPRIOR (sampling location as a priori) model performs well when no clear signal of a structure is detected or when there is low genetic differentiation, limited loci or a limited sample size⁷⁰. The simulations were run for 10⁵ burn-in and 10⁶ Markov chain Monte Carlo iterations (MCMC). The optimum number of K was inferred using the likelihood distribution $L(K)$ and the delta K' ⁷¹, which were estimated using the web version of Structure Harvester, v0.6.94⁷². The assignment plot was prepared using the program Distruct v1.1⁷³. Additionally, we used the DAPC, a multivariate nonmodel-based approach, to identify and describe genetic clusters⁴⁷. The optimal number of the clusters was estimated based on the lowest associated BIC. The analysis was performed using the *adgenet* in R studio.

We used BayesAss v3.0 to estimate the recent migration rate (m) between Chambal and Girwa populations⁷⁴. The simulations ($n = 3$) were run with different seed numbers using 10⁷ MCMC iterations and 10⁶ burn-in periods.

Bottleneck detection. We estimated demographic changes using two qualitative approaches: (a) the Garza–Williamson index (or M ratio) implemented in Arlequin v3.1⁷⁵ and (b) the HET approach implemented in Bottleneck v1.2.02⁷⁶. The M ratio estimates the ratio of the observed number of alleles to the size of allele range based on the assumption that in a recently reduced population, the ratio is expected to decrease due to random loss of alleles in a population. The calculated M ratio was then compared with the critical value ($M_C = 0.68$). An M ratio below M_C is considered as a signature of genetic bottleneck⁷⁷. The HET approach assumes that in a recently reduced population, an excess of the gene diversity under Hardy–Weinberg equilibrium is expected relative to gene diversity under mutation–drift equilibrium. We used one-tailed Wilcoxon test to determine the presence of a significant number of loci with excess heterozygosity. The estimates were calculated under two mutation models: the SMM and TPM. The TPM tends to be the most appropriate mutation model for microsatellite loci⁷⁸. The TPM was carried out at 95% SMM (variance at 12), and the simulations were run for 10⁴ iterations⁷⁶.

Mitochondrial DNA variation. We generated 63 mitochondrial CR sequences from the Girwa population and submitted it to GenBank (Accession No. MT500792–MT500854). We also obtained previously published mitochondrial CR sequences of the Chambal population ($n = 103$) from GenBank (Accession No. MT458816–MT458918)²². The sequences were aligned using the ClustalW algorithm⁷⁹ in BioEdit v7.2.6⁸⁰. The summary statistics, including the number of haplotypes, haplotype and nucleotide diversity, were estimated using DnaSP v5.10.01⁸¹.

Data availability

The mitochondrial sequences used in this study have been submitted to GenBank (Accession Nos. MT500792–MT500854). The microsatellite genotyping data used in the study is available on request from the corresponding author.

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Author contributions

S.A.H. conceived the study, raised funds and supervised the study and did review and editing of the manuscript. S.P.S. designed the methodology, collected samples, performed the experiment, data curation, data analysis and wrote the original manuscript. M.G.G. did data curation, data analysis and contributed in writing. N.D. contributed in writing and editing of the manuscript. S.K. collected samples and contributed in writing. S.K.G. and S.M. facilitated the validation and interpretation of the data. All authors agreed and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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Microsatellite analysis reveals low genetic diversity in managed populations of the critically endangered gharial (*Gavialis gangeticus*) in India

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The gharial (*Gavialis gangeticus*) is a critically endangered crocodylian, endemic to the Indian subcontinent. The species has experienced severe population decline during the twentieth century owing to habitat loss, poaching, and mortalities in passive fishing. Its extant populations have largely recovered through translocation programmes initiated in 1975. Understanding the genetic status of these populations is crucial for evaluating the effectiveness of the ongoing conservation efforts. This study assessed the genetic diversity, population structure, and evidence of genetic bottlenecks of the two managed populations inhabiting the Chambal and Girwa Rivers, which hold nearly 80% of the global gharial populations. We used seven polymorphic nuclear microsatellite loci and a 520 bp partial fragment of the mitochondrial control region (CR). The overall mean allelic richness (A_r) was 2.80 ± 0.40 , and the observed (H_o) and expected (H_e) heterozygosities were 0.40 ± 0.05 and 0.39 ± 0.05 , respectively. We observed low levels of genetic differentiation between populations ($F_{ST} = 0.039$, $P < 0.05$; $G'_{ST} = 0.058$, $P < 0.05$ Jost's $D = 0.016$, $P < 0.05$). The bottleneck analysis using the M ratio (Chambal = 0.31 ± 0.06 ; Girwa = 0.41 ± 0.12) suggested the presence of a genetic bottleneck in both populations. The mitochondrial CR also showed a low level of variation, with two haplotypes observed in the Girwa population. This study highlights the low level of genetic diversity in the two largest managed gharial populations in the wild. Hence, it is recommended to assess the genetic status of extant wild and captive gharial populations for planning future translocation programmes to ensure long-term survival in the wild.

The order Crocodylia is represented by 27 species and three families: Alligatoridae, Crocodylidae and Gavialidae¹. India harbours three crocodylian species: the gharial (*Gavialis gangeticus*), the mugger or marsh crocodile (*Crocodylus palustris*), and the saltwater crocodile (*Crocodylus porosus*). The gharial has the narrowest distribution range and is the most threatened species among all other crocodylian species occurring in the Indian subcontinent. Until the early twentieth century, the gharial was widely distributed in the Indus, Ganges, Mahanadi, Brahmaputra, Kaladan and Irrawaddy River systems spanning across Pakistan, India, Nepal, Bangladesh, Bhutan, and Myanmar^{2–4}. Over the years, the gharial has suffered a population decline of over 80% and substantial range contraction due to habitat loss, poaching, and mortalities in passive fishing^{5–7}. The species is now extinct in Myanmar, Bhutan and Pakistan. The extant gharial population is restricted to a few major river systems in India, Nepal and Bangladesh. It is presently listed as 'critically endangered' on the IUCN Red List of threatened species and is highly conservation dependent⁷.

In India, by the early 1970s, the gharial was restricted to few isolated locations in the Ganga, Mahanadi and Brahmaputra River systems². Due to the alarming decline in the gharial populations throughout its range, the Government of India listed it in Schedule I of the Indian Wild Life (Protection) Act, 1972 to provide enhanced protection. Subsequently, conservation translocation programmes, including reintroduction and restocking, were initiated in 1975 to restore its population in the wild⁵. As a part of these programmes, eggs collected from

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the wild were hatched and reared in rehabilitation centres, and individuals after attaining a length of 1.2 m were translocated into suitable habitats within the newly created protected areas^{5,6}.

In India, the head-start gharial conservation programme was initiated at the Chambal, Girwa and Mahanadi Rivers, where adult breeding populations existed. Initially, four facilities were established, viz. (1) Gharial Rehabilitation Centre, Tikarpada; (2) Gharial Rehabilitation Centre, Katarniaghat; (3) Gharial Rehabilitation Centre, Kukrail; and (4) Deori Gharial Rearing Centre, Morena targeting the Mahanadi, Girwa and Chambal populations. Hatchlings reared at these centres were released at the targeted rivers. In the Kukrail Centre, gharial hatchlings from 240 eggs collected from Chambal and 38 eggs collected from Girwa were reared during 1975^{8,9}. Although there is no record, the hatchlings from these two rivers were likely intermixed while rearing. The hatchlings produced in Kukrail and Deori were also translocated to several other rivers, viz. Son, Ken and Ramganga, some of which have started breeding¹⁰. Eggs were also collected from the Narayani River in Nepal, and hatchlings were released in the Girwa river. Additionally, conservation breeding was initiated in several zoos, such as the Nandankanan Biological Park and Madras Crocodile Bank, which contributed stocks for translocation¹¹. A male gharial from the Frankfurt Zoo, Germany, was obtained, which also contributed to developing stocks in Odisha for translocation¹². All translocation sites are now protected as wildlife sanctuaries under the Indian Wild Life (Protection) Act, 1972. In the last four decades, over 5000 gharial individuals have been translocated in more than 12 rivers in India, with over 3500 individuals in the Chambal and Girwa Rivers¹². At the beginning of the restocking programme, the gharial population in the Chambal River was 107 individuals, and now, it is approximately 1675 individuals of different ages and sexes^{4,13}. The Chambal River within the National Chambal Sanctuary (425 km) has been a stronghold of the gharial population since the initiation of the translocation programme. The Girwa River originally had a small population of approximately 34 individuals of different ages and sexes¹¹ and now has 43 adult individuals restricted to a 20 km stretch of the Girwa River falling within the Katarniaghat Wildlife Sanctuary. Presently, these two populations hold nearly 80% of the extant wild populations of gharial⁷.

Effective translocation programmes of endangered species essentially depend on prior knowledge of the ecological role, availability of suitable habitat, climatic requirements, behaviour, life-history traits, adequate post-release monitoring, demographic, behavioural and ecological aspects^{14–16}. The effectiveness of any translocation programme is also influenced by the level of genetic diversity of the source population¹⁷, affecting individual and population fitness, resilience against environmental change and long-term persistence¹⁸. However, most translocation programmes rarely evaluate this factor¹⁷. Therefore, understanding the genetic status of the translocated population is vital for determining the effectiveness of translocation. Since the initiation of the gharial conservation programme, several studies have been carried out to determine their ecological features^{2,4,6,13,19–21}. Nevertheless, studies on the genetic status of wild and captive gharial populations are limited^{22,23}.

In this study, we aim to assess the genetic diversity, population genetic structure and evidence of genetic bottlenecks using nuclear microsatellite loci and the mitochondrial control region (CR) of the two largest managed populations of gharials inhabiting the Chambal and Girwa Rivers (Fig. 1) to answer following research questions: (a) Do the two largest managed gharial populations differ in terms of genetic diversity? (b) Do the Chambal and Girwa River populations have their own genetic signatures? (c) If so, what is the level of differentiation? and (d) Did the demographic decline suffered by gharial populations in the last century also induce a genetic bottleneck?

Results

Microsatellite selection, screening and genotyping. Out of the 27 nuclear microsatellite loci (11 species-specific and 16 cross-species) screened, 18 loci showed successful amplification, and nine loci failed to amplify using known gharial samples (see Supplementary Table S1 online). We successfully genotyped 18 loci for 348 samples (Chambal = 232; Girwa = 116). Furthermore, out of 18 loci, 11 loci were found to be monomorphic and thus removed from further analyses. Overall, we used microsatellite genotype data of seven polymorphic loci from 348 gharial samples for population genetics analyses.

Genotyping quality and error rates. The polymorphic loci average amplification success rate was 96.09% for Chambal samples and 95.38% for Girwa samples. The quality index across the seven polymorphic loci was 0.86 ± 0.01 (mean $\pm$ SE) in Chambal and 0.89 ± 0.01 in Girwa (see Supplementary Figs. S1 and S2 online). The average allelic dropout (ADO), false allele (FA) and null allele frequency across polymorphic loci were below 5% in both populations (see Supplementary Table S2 online). We could not detect the occurrence of large allele dropout in our data.

Genetic variation. The cumulative probability of identity (PID biased) value of the panel of seven polymorphic markers was 1.74×10^{-3} , and the probability of identity (PID sibs) was 4.84×10^{-2} . We identified a total of 228 (Chambal = 162; Girwa = 66) distinct individuals using multilocus genotype data. The genetic diversity estimates for the Chambal and Girwa populations are summarised in Table 1. The number of alleles observed at each locus ranged from 2 to 7. The overall mean allelic richness was 2.80 ± 0.40 , and the observed (H_o) and expected (H_e) heterozygosities were 0.40 ± 0.05 and 0.39 ± 0.05 across seven polymorphic loci, respectively. The mean allelic richness estimated using the rarefaction approach was 2.91 ± 0.60 in the Chambal population and 2.48 ± 0.48 in the Girwa population. The mean observed and expected heterozygosities were 0.42 ± 0.07 and 0.41 ± 0.06 in the Chambal and 0.42 ± 0.08 and 0.42 ± 0.09 in the Girwa populations, respectively. The inbreeding coefficient (F) was -0.03 for both populations. None of the loci showed significant deviation from HWE following Bonferroni correction.

Population genetic structure, differentiation and migration. The Bayesian approach implemented in Structure v2.3.4 identified two ($K=2$) optimum number of clusters inferred by the likelihood distribution

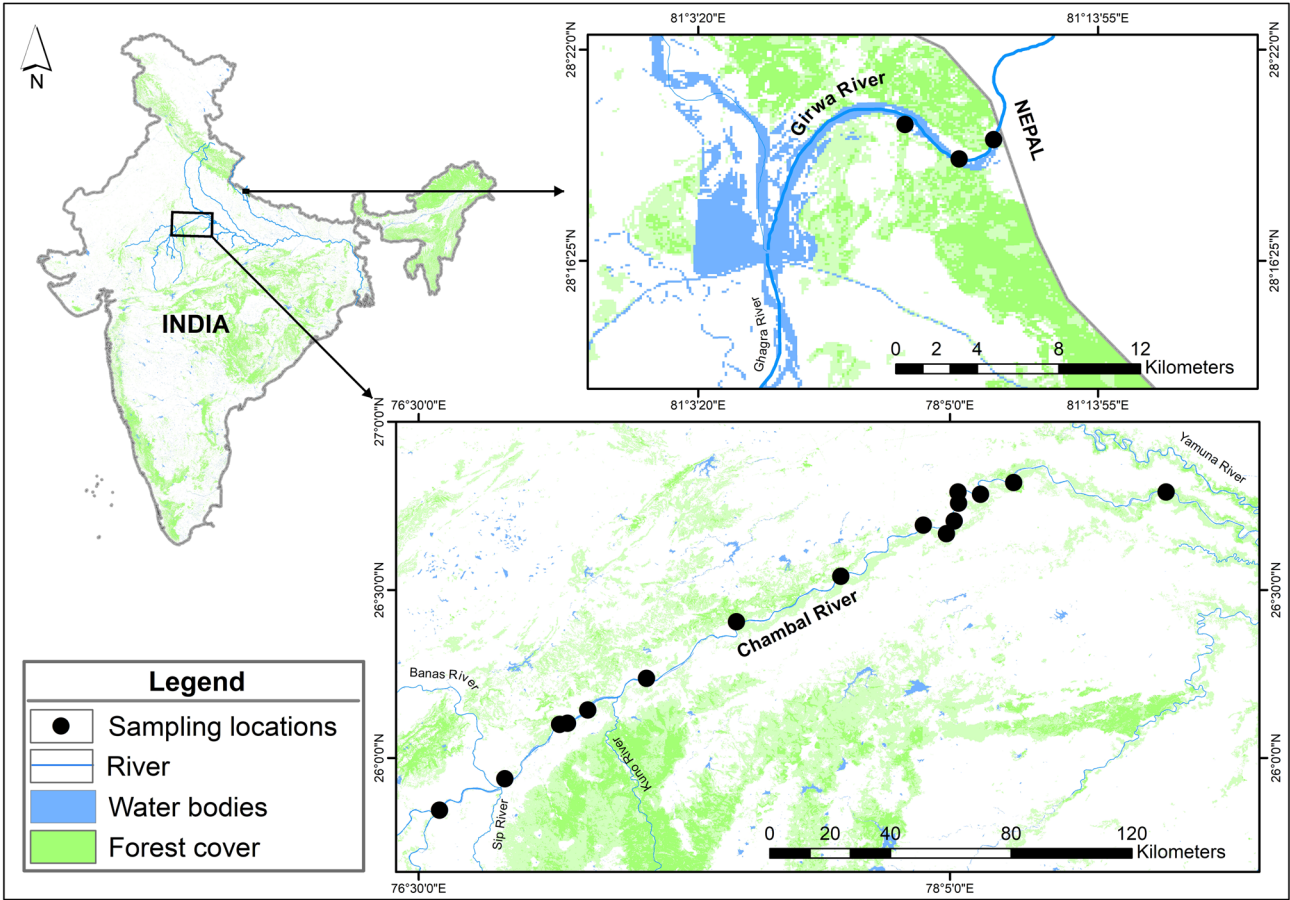


Figure 1. Map showing detailed representation of the study area and sampling locations along the Chambal and Girwa Rivers. The map was prepared using ArcGIS v.10.3.1 software developed by ESRI (<https://www.esri.com>).

Population	Chambal river						Girwa river					
Locus	N	Na	Ar	Ho	He	F	N	Na	Ar	Ho	He	F
G13_7	160	2	2.0	0.31	0.33	0.07	66	2	2.0	0.50	0.49	−0.02
Cj16	158	2	2.0	0.27	0.27	−0.01	66	2	2.0	0.03	0.03	−0.02
G13_2	162	2	2.0	0.54	0.50	−0.09	66	2	2.0	0.56	0.48	−0.17
G13_5	153	3	3.0	0.24	0.23	−0.05	Monomorphic					
G13_8	159	7	6.3	0.74	0.67	−0.11	66	5	5.0	0.53	0.63	0.16
G13_14	160	3	3.0	0.42	0.42	0.00	66	3	3.0	0.42	0.52	0.19
G13_16	160	2	2.0	0.44	0.44	−0.01	66	2	2.0	0.47	0.36	−0.31
Mean		3	2.91	0.42	0.41	−0.03		2.67	2.48	0.42	0.42	−0.03
SE		0.69	0.60	0.07	0.06	0.02		0.49	0.48	0.08	0.09	0.08

Table 1. Genetic diversity of 228 individuals at seven microsatellite nuclear loci. Number of individuals sampled (N), number of alleles per locus (Na), standardised number of alleles (Ar), observed heterozygosity (Ho), expected heterozygosity (He), Inbreeding coefficient (F).

$L(K)$ and ΔK estimates (see Supplementary Fig. S3 online). All Girwa samples were assigned to cluster-I, with an average proportion of membership (q) of 0.97, and Chambal samples were assigned to cluster-II, with $q = 0.82$ (Fig. 2a). Genetic differentiation measures were low but significant as derived using both the fixation indices: $F_{ST} = 0.039$ ($P < 0.05$); $G'_{ST} = 0.058$ ($P < 0.05$) and allelic differentiation index Jost's $D = 0.016$ ($P < 0.05$).

The discriminate analysis of principal component (DAPC) identified 20 genetic clusters associated with the lowest Bayesian information criterion (BIC) (see Supplementary Fig. S4 online). All the identified clusters ($K = 20$) showed an overlapping pattern with no clear structuring (Fig. 2b). Furthermore, the recent migration rate (m) was 0.12 (95% CI 0–0.26) from Chambal to Girwa River and 0.02 (95% CI 0–0.05) from Girwa to Chambal.

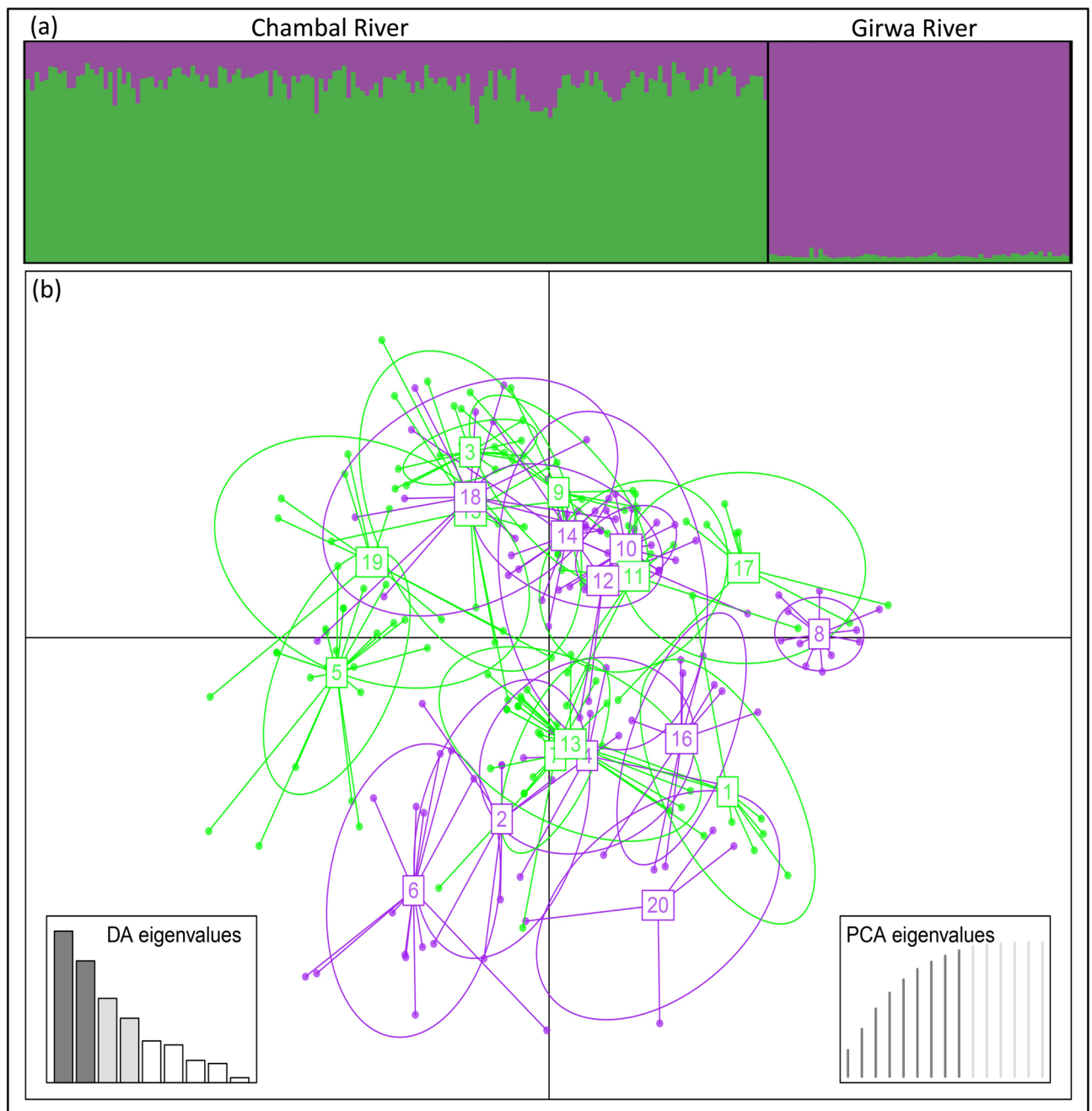


Figure 2. (a) Population genetic structure of 228 gharial individuals derived using a Bayesian clustering approach implemented in STRUCTURE. A single bar plot represents the individual, and the extent of colour in each bar indicates the probability of assigning the individual to a particular cluster. (b) Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of 228 gharial individuals. This scatterplot shows the first two principal components of the DAPC.

Bottleneck. The M ratio calculated across polymorphic loci was 0.31 ± 0.06 in Chambal and 0.41 ± 0.12 in Girwa, indicating a genetic bottleneck in both populations. The heterozygosity excess test (HET) using the stepwise mutation model (SMM) (Chambal $P=0.28$; Girwa $P=0.08$) and the two-phase model (TPM) (Chambal $P=0.18$; Girwa $P=0.08$) failed to detect the signature of a genetic bottleneck. We also obtained a normal L-shaped allelic distribution for both populations.

Mitochondrial DNA variation. We observed two mitochondrial CR haplotypes H1 in 58 individuals (92%) and H2 in five individuals (8%) with a single parsimony-informative site (at the 375-nucleotide position) in the Girwa population. Haplotype, H1 was shared by both the Girwa and Chambal populations²², whereas H2 was unique to the Girwa population. The haplotype diversity was (mean $\pm$ SD) 0.148 ± 0.057 , and nucleotide diversity was 0.00029 ± 0.00011 in the Girwa population.

Discussion

This study is the first comprehensive genetic assessment of the critically endangered gharial and provides crucial baseline information essential for guiding the ongoing conservation efforts. The genetic diversity observed at seven polymorphic nuclear microsatellite loci was low in the gharial populations of Chambal and Girwa. Both populations had similar mean heterozygosities, and the allelic richness (Ar) was marginally higher in the Chambal population (Table 1). The overall heterozygosities at nuclear loci in the wild ($H_o = 0.40 \pm 0.05$; $H_e = 0.39 \pm 0.05$) were lower than the previously reported estimates in captive ($H_o = 0.92 \pm 0.02$; $H_e = 0.65 \pm 0.02$) gharial populations using the same set of microsatellite loci. The estimated mean allelic richness was also lower in the wild population (Chambal = 2.91 ± 0.60 ; Girwa = 2.48 ± 0.48) than that reported in captivity (5.5 ± 0.5)²³. A low level of heterozygosity is not rare in wild crocodylians. Similar levels of heterozygosity have been reported in other crocodylians, including *Alligator sinensis*²⁴, *Alligator mississippiensis*²⁵, *Caiman yacare*²⁶, *Crocodylus siamensis*²⁷, *Crocodylus mindorensis*²⁸, *Crocodylus moreletii*^{29,30}, and *Crocodylus palustris*^{31,32}, as well as in other vertebrate species that have experienced demographic bottlenecks³³. Moreover, the Girwa population exhibits two and Chambal population exhibits a single mitochondrial CR haplotype²². The presence of extremely low variation at a hypervariable locus is surprising because, in other crocodylian species, the reported haplotypic diversity at mitochondrial CR is considerably higher^{34–38}. The genetic diversity of a species is influenced by its geographic range, abundance, demography, and life-history traits. Narrow-ranging, less abundant and long-lived species with demographically challenged population history tend to have lower genetic diversity than widely distributed, abundant species with stable demographic history^{39–41}. The low abundance, narrow distribution range, complex life-history traits, and unstable demography of the gharial may have resulted in the observed low level of genetic diversity.

The coefficient of inbreeding (F), primarily measures the deviation from HWE (where $F = 0$ indicates that the population is at HWE) and is often only weakly correlated with inbreeding and fitness^{42,43}. The estimate is likely to be affected by several factors including use of a limited number of loci, closely related individuals, genetic bottleneck, gene flow and admixture^{44–46}. Additionally, populations that experience demographic bottlenecks do not necessarily become inbred. Our estimate of $F \approx 0$, shows that the populations are at or near HWE.

Population genetic structure, differentiation, and migration. The presence of population genetic structure was supported by Structure (Fig. 2a) and genetic differentiation indices ($F_{ST} = 0.039$, $P < 0.05$; $G'_{ST} = 0.058$, $P < 0.05$ Jost's $D = 0.016$, $P < 0.05$) but not by multivariate analysis DAPC (Fig. 2b). The Bayesian approach implemented in Structure is a model-based approach that largely depends on the assumption of population genetics models. In contrast, DAPC is a model-free approach that does not rely on population genetics models. DAPC performs better than Structure in characterizing population clusters⁴⁷. Therefore, we believe that the result of DAPC is more reliable and appropriate for our dataset. The observed low level of differentiation and an admixed population structure suggested by DAPC hint towards possible intermixing of gharial individuals during the translocation programme when eggs from both the Chambal and Girwa Rivers were reared in the Kukrail centre.

Furthermore, we observed a migratory relation between the two populations. This supports our hypothesis of possible mixing of individuals during the translocation programme. It is important to note that the observed migration rate from Chambal to Girwa was higher than that from Girwa to Chambal, which is possible due to large number of eggs were sourced from Chambal and later released in the Girwa River. We could not perform genetic structure, differentiation, and migration analyses using mitochondrial CR because of low variability in the analysed region.

Bottleneck. The estimates of the M ratio (Chambal = 0.31 ± 0.06 ; Girwa = 0.41 ± 0.12) were lower than the critical value ($M_C = 0.68$), confirming the signature of genetic bottlenecks in both populations. In contrast, the HET failed to detect the signature of a genetic bottleneck. This discrepancy between the results could be due to the limited statistical power of the two analyses. The M ratio can effectively detect genetic bottlenecks for a greater amount of time (up to 50 generations), whereas HET can efficiently detect bottleneck that occurred within a short time, $0.2–4.0 N_e$ (effective population size) generations^{48,49}. The gharial population has suffered two instances of range-wide population decline during the early-1970s and mid-1990s, when the population dwindled to less than 200 adult individuals in the wild^{6,20}. The average generation time estimates for gharial individuals is 25 years⁷, suggested that the latter decline in population occurred very recently (approx. 1–1.5 generation ago). Hence, the undetectability of the bottleneck through HET could be due to the recent bottleneck event. Additionally, bottleneck detection can be masked due to various confounding factors including time of occurrence, duration, magnitude, gene flow, pre-bottleneck genetic variability, mating system, sample size and a limited number of loci^{50–52}. Hence, there is a need to investigate both of these populations with extensive microsatellite loci to achieve high statistical power^{49,53,54}.

In conclusion, low polymorphism was observed in species-specific and cross-species microsatellite loci; it remains crucial to develop novel microsatellite loci for population genetic studies. We acknowledge that our study might have been influenced by the inclusion of closely related individuals from each nesting site. However, the sampling was conducted from a large number of nests spread across 19 nesting sites. Therefore, we believe that our sampling strategy potentially represents the level of genetic diversity of the studied populations and minimises the bias arising from closely related individuals.

Conservation implication. Gharial translocation is considered as one of the most successful species recovery programmes in the world¹² in terms of increasing the abundance of the species in the wild. However, despite a long history of conservation efforts, limited information on the genetic status of the gharial has hindered, if

not precluded, effective conservation planning. Gharial populations inhabit different biogeographic zones of India and are adapted to specific environmental conditions, yet translocation programmes have not considered the effects of genetic intermixing among populations. Such intermixing of populations without prior knowledge of the genetic structure can potentially threaten the genetic integrity and distinctiveness of the resident populations¹⁴. Our study augments the existing knowledge on the genetic status of the critically endangered gharial, which is vital for future translocations and research. This study highlighted low levels of genetic diversity and admixed structure in the two largest managed populations of gharial in the wild, which are considerably lower than the previously reported estimates of captive populations²³.

Moreover, the gharial is a highly conservation-dependent species, and translocations without assessing the genetic status of the source populations may further deteriorate the level of genetic diversity in the translocated populations. Hence, we recommend limiting the interpopulation release of individuals to prevent further intermixing of the gene pool until information on the genetic status of the extant wild and captive gharial populations using an extensive dataset of mitochondrial and microsatellite markers is available. Information on the genetic status will assist in the identification of potential source populations and maintain adequate levels of genetic diversity to secure the continuing persistence of gharials in the wild.

Methods

Research permits and ethical considerations. The Forest Department of Madhya Pradesh (Letter No. SL/Tech-1/8200), Rajasthan (Letter No. 1399) and Uttar Pradesh (Letter No. 3093 and 3778(A)) provided all required permissions for the survey and collection of the biological samples. As all samples were collected from either dead remains of hatchlings or hatched eggshells, no animal ethical clearance was required for this study.

Sample collection and DNA extraction. Sampling of a large number of adult individuals required for population genetic assessments has its own ethical and logistical constraints, especially when the species in question is critically endangered. Considering these restrictions, we collected tissue from the remains of dead hatchlings and chorioallantoic membranes from hatched eggshells shortly after hatching. Samples were collected from 16 nesting sites along the Chambal River and three nesting sites along the Girwa River during 2017 and 2018, respectively (Fig. 1). We collected 348 samples (Chambal = 232 samples and Girwa = 116 samples) for genetic assessment (see Supplementary Table S3 online). Out of 348 samples, 49 samples were unique (one sample per clutch), 138 samples were from a sibling (more than one sample per clutch), and 161 samples had no clutch information (see Supplementary Table S4 online). All samples were stored in absolute ethanol at room temperature and later at -20°C in the laboratory for long-term storage. Total genomic DNA was extracted from tissue (64 ± 19 mg) (mean $\pm$ SD) and chorioallantoic membrane using the phenol–chloroform method⁵⁵. Cotton swabs were used when chorioallantoic membrane was unable to separate from eggshells.

Microsatellite selection, screening and genotyping. We initially screened 11 of the 18 gharial specific microsatellite loci described by Jogayya et al.²³. However, only six of the eleven loci were found to be polymorphic. Hence, we conducted an exhaustive literature survey to identify the potential cross-species loci used across families Alligatoridae, Crocodylidae and Gavialidae. We found a total of 424 previously described microsatellite loci and listed the loci based on successful cross-species transferability, allele diversity, heterozygosity, polymorphic information content and allele range. Finally, we selected 16 cross-species loci developed in eight different crocodylian species for screening. Out of 16, a total of 12 loci were selected based on successful cross-species transferability in other crocodylian species. The remaining four loci were selected based on a number of alleles ≥ 4 , H_o and $H_e \geq 5$, $PIC \geq 0.5$ and allelic range < 300 bp. The list of microsatellite loci screened in the current study is provided in Supplementary Table S1 online.

Polymerase chain reactions (PCR) were performed in 10 μL reaction volumes containing 5 μL of 2 $\times$ QIAGEN Multiplex PCR master mix, 2 μL of 5 $\times$ Q-solution (QIAGEN Inc., Germany), and the labeled forward primer at 0.15 μM and the reverse primer at 0.15 μM and 2 μL of DNA template. The thermal profiles included an initial denaturation at 95°C for 15 min, followed by 35 cycles at 94°C for 40 s, T_a at 56 – 62°C (see Supplementary Table S1 online) for 60 s, 72°C for 60 s and a final extension of 72°C for 30 min. The amplified products were genotyped using the GeneScan 500 LIZ dye size standard (Applied Biosystems) in 3500XL Genetic Analyzer (Applied Biosystems). The alleles were scored using the program GeneMarker v2.7.4 (SoftGenetics, LLC) with combined automated allele scoring and validated through visual inspection. Three replicates of each sample were carried out to obtain reliable multilocus genotypes using noninvasive samples following a multiple-tube approach⁵⁶.

Mitochondrial CR DNA sequencing. We selected a 520 bp partial fragment mitochondrial CR to assess the genetic variation in gharials. We used primers (L15637 5'-GCATAACACTGAAAATGTTAAAYATGG-3' and H16258 5'-CTAAAATTACAGAAAAGCCGACCC-3') described by Oaks (2011) to amplify the selected fragment⁵⁷. The PCRs were carried out in 20 μL volumes containing 2 μL of the DNA template, 10 μL of 2 $\times$ PCR buffer, 0.2 mM of dNTPs, 0.25 μL of each primer, and 0.1 μL (0.5 units) of DreamTaq DNA polymerase (Thermo Scientific). The thermal profile was 95°C for 5 min, followed by 35 cycles at 95°C for 35 s, 56°C for 40 s, 72°C for 45 s, and a final extension of 72°C for 10 min. The amplified products were visualised using a 2% agarose gel. The positive amplicons were cleaned up with Exonuclease-I (Thermo Scientific) and Shrimp Alkaline Phosphatase (Applied Biosystems), and sequenced using forward primers in 3500XL Genetic Analyzer (Applied Biosystems).

Data analysis. Genotyping quality and error rates. We obtained a consensus genotype and estimated the quality index for each locus genotyped following Miquel et al.⁵⁶. We scored each repeat with '1' if the genotype

was identical to the consensus and '0' if the genotype did not match the consensus due to any errors such as no amplification, allelic dropout, or false allele⁵⁶. We estimated the average amplification success as a percent of positive PCR amplification. We estimated the frequency of genotyping errors due to allelic dropout (ADO) and false allele (FA) following Broquet et al.⁵⁸. The frequency of null alleles was estimated using FreeNa⁵⁹ and occurrences of large allelic dropout using Micro-Checker v2.2.3⁶⁰.

We used samples with a quality index above 0.67 per locus (identical genotypes in two replicates out of three replicates) and a mean quality index across loci above 0.75 for analyses.

Genetic variation. We calculated the probability of identity PID (biased) and PID (sibs) using Gimlet v1.3.3⁶¹. We estimated summary statistics (number of alleles per locus, observed heterozygosity, expected heterozygosity, and inbreeding coefficient) using GenAlEx v6.0⁶² and deviations from Hardy–Weinberg equilibrium (HWE) for each locus using Bonferroni correction in Cervus v3.0.7⁶³. We also estimated allelic richness using a rarefaction approach implemented in HP-Rare v1.1 to account for the uneven sample size of the two populations⁶⁴.

Population structure, differentiation, and migration. We have estimated the genetic differentiation using fixation indices F_{ST} ⁶⁵, G'_{ST} ⁶⁶ and allelic differentiation index Jost's D' ⁶⁷ using the *strataG* package⁶⁸ implemented in R studio. The estimates were calculated using 10³ bootstrap iterations. We inferred the population genetic structure using the Bayesian approach implemented in Structure v2.3.4⁶⁹. Structure is a systematic model-based Bayesian clustering approach that uses allele frequencies at each locus to infer the population structure. The analysis was performed for 1–10 clusters (K). For each K , ten iterations were run under the admixture model with correlated allele frequencies and sampling location as a priori. The LOCPRIOR (sampling location as a priori) model performs well when no clear signal of a structure is detected or when there is low genetic differentiation, limited loci or a limited sample size⁷⁰. The simulations were run for 10⁵ burn-in and 10⁶ Markov chain Monte Carlo iterations (MCMC). The optimum number of K was inferred using the likelihood distribution $L(K)$ and the delta K' ⁷¹, which were estimated using the web version of Structure Harvester, v0.6.94⁷². The assignment plot was prepared using the program Distruct v1.1⁷³. Additionally, we used the DAPC, a multivariate nonmodel-based approach, to identify and describe genetic clusters⁴⁷. The optimal number of the clusters was estimated based on the lowest associated BIC. The analysis was performed using the *adgenet* in R studio.

We used BayesAss v3.0 to estimate the recent migration rate (m) between Chambal and Girwa populations⁷⁴. The simulations ($n = 3$) were run with different seed numbers using 10⁷ MCMC iterations and 10⁶ burn-in periods.

Bottleneck detection. We estimated demographic changes using two qualitative approaches: (a) the Garza–Williamson index (or M ratio) implemented in Arlequin v3.1⁷⁵ and (b) the HET approach implemented in Bottleneck v1.2.02⁷⁶. The M ratio estimates the ratio of the observed number of alleles to the size of allele range based on the assumption that in a recently reduced population, the ratio is expected to decrease due to random loss of alleles in a population. The calculated M ratio was then compared with the critical value ($M_C = 0.68$). An M ratio below M_C is considered as a signature of genetic bottleneck⁷⁷. The HET approach assumes that in a recently reduced population, an excess of the gene diversity under Hardy–Weinberg equilibrium is expected relative to gene diversity under mutation–drift equilibrium. We used one-tailed Wilcoxon test to determine the presence of a significant number of loci with excess heterozygosity. The estimates were calculated under two mutation models: the SMM and TPM. The TPM tends to be the most appropriate mutation model for microsatellite loci⁷⁸. The TPM was carried out at 95% SMM (variance at 12), and the simulations were run for 10⁴ iterations⁷⁶.

Mitochondrial DNA variation. We generated 63 mitochondrial CR sequences from the Girwa population and submitted it to GenBank (Accession No. MT500792–MT500854). We also obtained previously published mitochondrial CR sequences of the Chambal population ($n = 103$) from GenBank (Accession No. MT458816–MT458918)²². The sequences were aligned using the ClustalW algorithm⁷⁹ in BioEdit v7.2.6⁸⁰. The summary statistics, including the number of haplotypes, haplotype and nucleotide diversity, were estimated using DnaSP v5.10.01⁸¹.

Data availability

The mitochondrial sequences used in this study have been submitted to GenBank (Accession Nos. MT500792–MT500854). The microsatellite genotyping data used in the study is available on request from the corresponding author.

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Author contributions

S.A.H. conceived the study, raised funds and supervised the study and did review and editing of the manuscript. S.P.S. designed the methodology, collected samples, performed the experiment, data curation, data analysis and wrote the original manuscript. M.G.G. did data curation, data analysis and contributed in writing. N.D. contributed in writing and editing of the manuscript. S.K. collected samples and contributed in writing. S.K.G. and S.M. facilitated the validation and interpretation of the data. All authors agreed and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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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)

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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 ($h_d = 0.462 \pm 0.048$; $\pi = 0.00029 \pm 0.00004$). 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).

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

Gene	Primer	Sequence	Ta (°C)	Source
<i>coxI</i>	FishF1	5'-TCAACCAACCACAAAGACATTGGCAC-3'	56	Ward et al. (2005)
	FishR1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'		
<i>cytb</i>	CP14715	5'-TGAGGAGCAACCGTAATTACCAACCT-3'	56	Meganathan et al. (2009)
	CP15546	5'-TCTGTCTTACAAGGCCAGTGCTTT-3'		
<i>CR</i>	L15637	5'-GCATAAACTGAAAATGTTAAYATGG-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

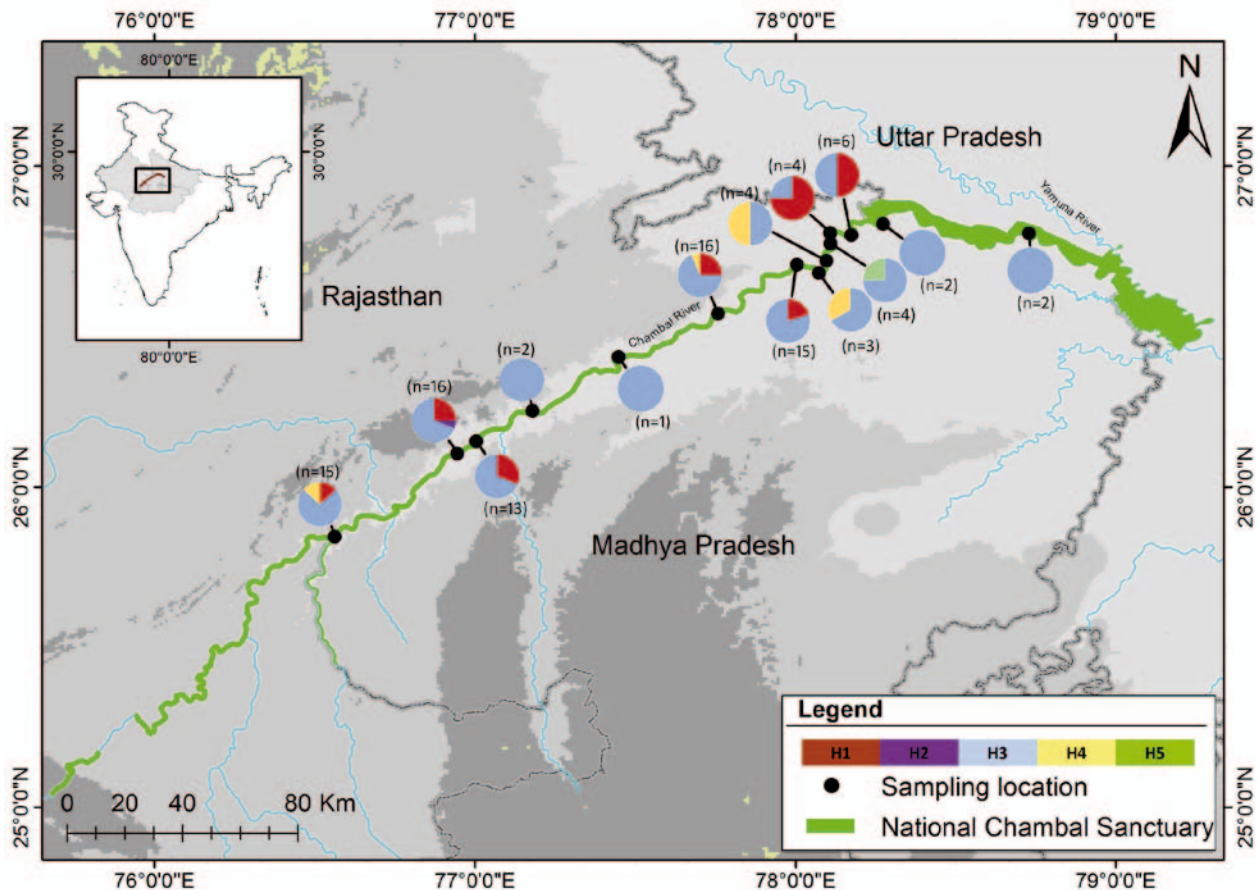


Figure 1. Map showing haplotype distribution of *G. gangeticus* in sampling locations. Pie charts represent the respective frequencies of mitochondrial DNA haplotypes in particular sampling location and n= number of samples. The colours of the pie charts represent different haplotypes.

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 (h_d , mean $\pm$ SD) was 0.462 ± 0.048 , and the nucleotide diversity (P_i , mean $\pm$ 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

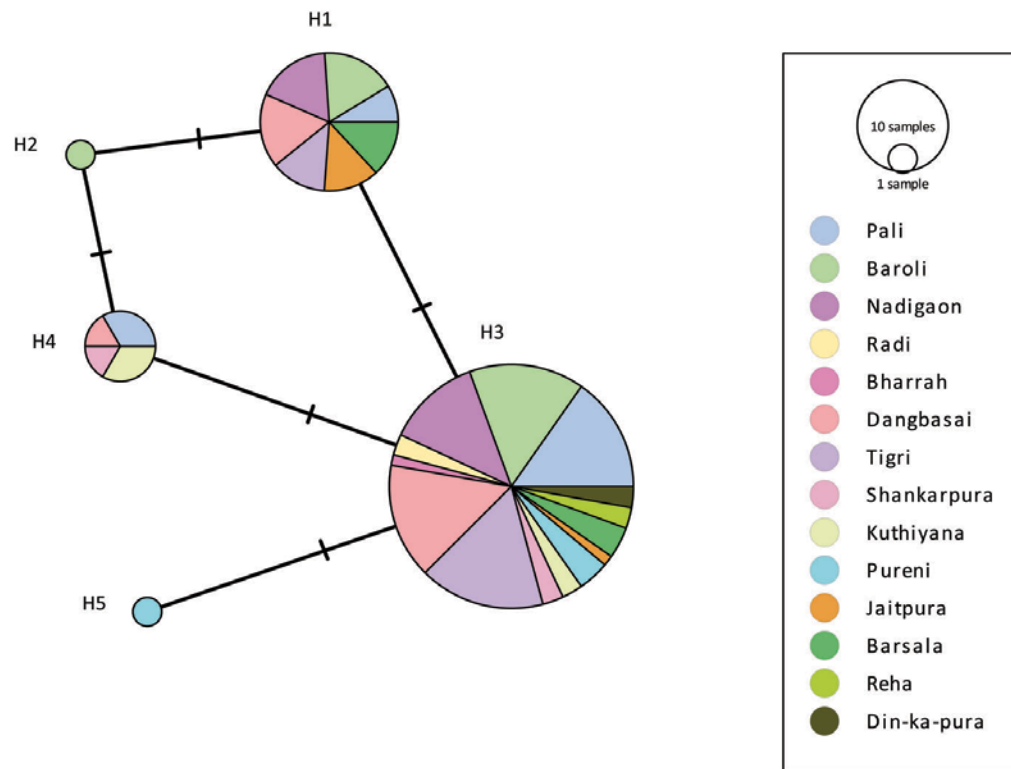


Figure 2. Median-joining haplotype network inferred from 1,806 bp of mtDNA sequence data for *G. gangeticus*. Each circle represents a different haplotype and the size is indicative of number of individuals present within the haplotype. The colours represent the proportions of the haplotypes from each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

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

Haplotype 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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