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Population genetics of gharial *Gavialis gangeticus* in the Chambal River, India, using novel polymorphic microsatellite markers

Ravi Kumar Singh[1,3], Jailabdeen AjjiM[2], Jeffrey W. Lang[2], Harika Segu[1], Harisharan Ramesh[1], Karthikeyan Vasudevan[1,3,*]

[1]CSIR –Centre for Cellular and Molecular Biology, Laboratory for the Conservation of Endangered Species, Hyderabad, Telangana 500048, India

[2]Gharial Ecology Project, Madras Crocodile Bank Trust, Mamallapuram, Tamil Nadu 603104, India

[3]Academy of Scientific and Innovation Research (AcSIR), Ghaziabad, Uttar Pradesh 201002, India

ABSTRACT: The gharial *Gavialis gangeticus* is a Critically Endangered crocodylian endemic to the Indian subcontinent. The species has experienced a 95% population decline over the past 2 centuries. The largest self-sustaining population inhabits the protected National Chambal Sanctuary (NCS) in north India and represents >80% of extant gharials globally. We developed de novo a panel of polymorphic gharial-specific microsatellites, using whole genome information and microsatellite search tools. These 15 new markers have multiple numbers of polymorphic alleles that are more informative than those obtained from previous studies. Analyses of 93 scute samples collected across age classes from wild gharials residing in the NCS facilitated accurate assessments of genetic diversity and inbreeding coefficient and identified a historical bottleneck event. Estimates of the observed and expected heterozygosities were lower than those reported earlier. The inbreeding coefficient was low, and the population did not deviate significantly from Hardy-Weinberg equilibrium. The calculated *M* ratio and 2 heterozygosity tests detected a genetic bottleneck, which is consistent with historic sharp declines in population size, followed by recent recovery. These new gharial microsatellite markers are statistically robust and provide an improved means to assess the population genetics of the largest self-sustaining wild gharial population. This study will facilitate additional investigations on the genetic diversity of other extant gharial populations — not only the few remaining wild populations but also those in zoos and rearing facilities. Additional genetic studies of gharial in the NCS are warranted to inform management strategies.

KEY WORDS: Crocodylian · SSR markers · Whole genome sequence · Genetic polymorphism · Population bottleneck · Long-lifespan species

1. INTRODUCTION

Surviving Crocodylia consists of ~28 species belonging to 3 families: Alligatoridae, Crocodylidae, and Gavialidae (Grigg & Krishner 2015, Colston et al. 2020). The gharial *Gavialis gangeticus* is one of 2 surviving members of Gavialidae. Gharials were once widely distributed in large South Asian rivers, such as the Indus, Ganges, Brahmaputra, Mahanadi, Kaladan and Irrawaddy, as well as their tributaries (Lang et al. 2019). Historically, the species has been recorded in large rivers in Pakistan, India, Nepal, Bangladesh, Bhutan, and Myanmar (Singh 1978, Whitaker & Basu 1983, Lang et al. 2019). Over the past 2 centuries, the gharial population has declined by 95% due to habitat loss, poaching, mortality in fishing nets, and habitat

*Corresponding author: karthik@ccmb.res.in

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fragmentation caused by large dams and water harvesting (Bustard 1975, Whitaker 2007, Stevenson 2015). Currently, the species is extirpated from a majority of the original range, and remnant populations survive in a few major rivers in India and Nepal. In 2007, its conservation status was assessed as Critically Endangered (Choudhury et al. 2007). Today, the largest self-sustaining population (>650 adults; ~2000 in total) inhabits the National Chambal Sanctuary (NCS) in India, along ~425 km of the Chambal River and several upstream tributaries (Lang et al. 2019)

Effective conservation translocations and reintroduction programs of endangered species are enhanced by knowledge of their demographic history and population genetics (Schwartz 2005, White et al. 2018). Specific information about population genetics is important for restocking because the genetic diversity of founder populations directly affects the conservation outcomes (Weeks et al. 2011). Gharials are a flagship for riverine conservation and are important for a healthy riverine ecosystem (Maskey et al. 2006, Behera et al. 2014). Previous actions to augment populations or repopulate riverine habitats have involved translocations and/or introductions, albeit without any prior genetic data (Storfer 1999). Like other crocodiles, the species is long-lived and highly fecund but is also highly mobile and exceptionally social (Lang & Kumar 2013, 2016). Most investigations have focused on the species' ecology (Singh 1978, Hussain 1999, Lang & Whitaker 2010, Nair et al. 2012, Lang & Kumar 2013, Vashistha et al. 2021, Griffith et al. 2023) and few have dealt with gharial population genetics (Jogayya et al. 2013, Green et al. 2014, Sharma et al. 2020, 2021, Vashistha et al. 2020).

Microsatellite markers have been extensively used for crocodylian genetic studies that have examined genetic diversity and population structure (Dever et al. 2002, De Thoisy et al. 2006, Hinlo et al. 2014, Velo-Antón et al. 2014, Muniz et al. 2019), paternity (Uller & Olsson 2008), kinship (Muniz et al. 2011, Budd et al. 2015), reduction in effective population size (N_e) (Bishop et al. 2009), and reintroduction and restocking (Rodriguez et al. 2011). In most of these reports, the focus has been on mating systems (Isberg 2022) and conservation and management (Amavet et al. 2021). Informative microsatellite markers are desirable for reliable population genetic investigations (Selkoe & Toonen 2006). Unfortunately, the population genetics of the NCS population remains poorly understood due to a lack of informative microsatellite markers, as evidenced in Sharma et al. (2021).

Our overall goal with respect to gharial population genetics is to perform analyses that are informative for conservation management. Our objectives in this study are (1) to present a novel, polymorphic panel of gharial-specific microsatellite markers and (2) to utilize these markers in a robust analysis of population-level genetic variation of wild-caught resident gharial in the Chambal River. An analysis of parentage relationships within and among nests at colony nesting sites is forthcoming elsewhere.

2. MATERIALS AND METHODS

2.1. Sample collection and DNA isolation

Gharials of all size classes were targeted for a telemetry study undertaken by the Gharial Ecology Project (GEP). Details of the samples used for the study are given in Table S1 in the Supplement at www.int-res.com/articles/suppl/n053p127_supp.pdf. A total of 100 tail scute samples were collected from wild-caught gharials between 2009 and 2017 (see Lang & Whitaker 2010) along a 100 km stretch of the NCS by members of the GEP (Fig. 1). Samples were stored long-term at −20°C. A subsample of the tissue was macerated and DNA was isolated using phenol-chloroform (Sambrook et al. 1989). Final concentrations were measured using NanoDrop™.

2.2. Development of microsatellite markers

2.2.1. Mining of microsatellite markers

We used the assembly-level genome GavGan_comp1 (accession no. GCA_001723915.1) from the genomic resources of the National Centre for Biotechnology Information Database (NCBI) (https://www.ncbi.nlm.nih.gov/genome) to mine the simple sequence repeat (SSR) markers. We downloaded the BioProject and RefSeq assembly (accession nos. PRJNA172383 and GCF_001723915.1) in FASTA format from NCBI Genome Assembly resources. The scaffold-level assembly of the genome had 81 scaffolds. The total length and coverage of the genome sequenced in the Illumina HiSeq platform were 2 640 792 433 bp (2.6 Gb) and 81.0×, respectively. We searched for perfect and compound SSRs using Krait (https://github.com/lmdu/krait; Du et al. 2018) for a genome-wide search of SSRs. We did not use imperfect SSRs, as they might be less polymorphic than perfect repeats due to reduced strand slippage (Ashley & Dow 1994). Owing to the large genome size, we increased the stringency of SSR

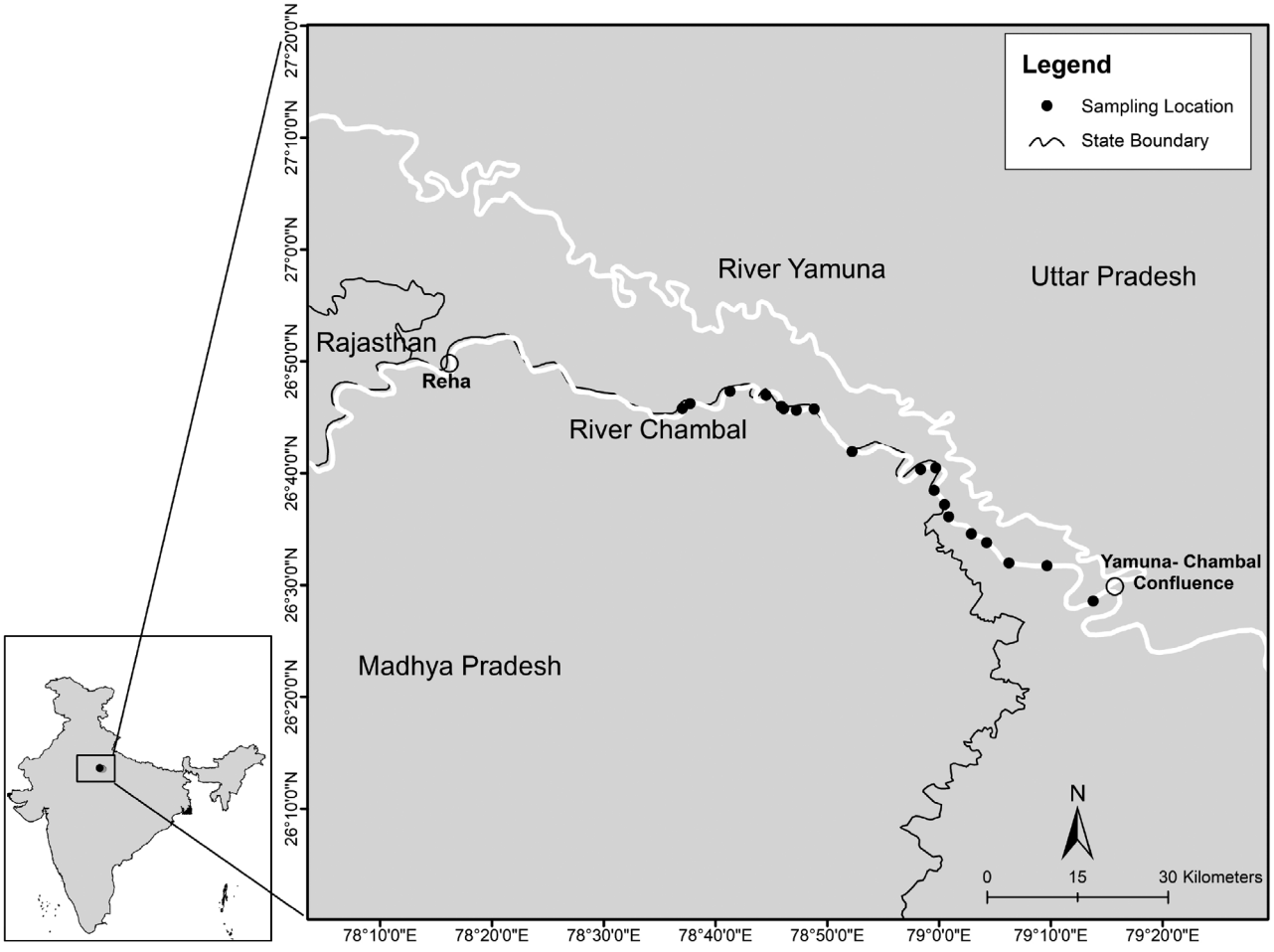

Fig. 1. Location of gharial samples collected in the National Chambal Sanctuary (NCS), North India. The river stretch shown spans roughly 160 km from Reha to the Yamuna–Chambal confluence. Inset map: location of the study area in India. Black dots: sample locations across NCS; dotted black line: state boundary of Uttar Pradesh and Madhya Pradesh

mining using search criteria that involved fixing the parameters of the minimum number of repeats at 30, 15, 10, 8, 6, and 5 for mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs (Matsubara et al. 2016, Liu et al. 2019a). Since the loci with a low number of repeats are usually less polymorphic (McDonald & Potts 1997), the minimum number of repeats for all the motifs was set high for the mining of SSR markers. The distance between the 2 SSRs (dMax) was set at 10 bp, so the interference or overlapping repeats could be excluded for compound SSRs. The repeat motifs were standardized based on Level 3 criteria (Matsubara et al. 2016, Liu et al. 2019a), in which similar motifs and reverse complementary motifs were grouped for statistical analysis. The length of the flanking sequences was set at 200 bp to enhance the efficiency of primers for the SSRs.

2.2.2. Primer designing and *in silico* PCR

Mononucleotide repeats amplify poorly and have low variability (Li et al. 2002). Dinucleotide repeats have a large stutter (>30%) and a low mutation rate (McDonald & Potts 1997). We did not use mononucleotide or dinucleotide repeats. In forensics and parentage analyses, trinucleotide and tetranucleotide repeats are preferred (Butler 2012, Vieira et al. 2016). Pentanucleotide and hexanuclotide repeats result in large PCR products and they might hinder PCR amplification and result in null alleles. Therefore, we selected only trinucleotide and tetranucleotide repeat SSRs to design the primer pairs. Primer pairs for all mined perfect SSRs having trinucleotide and tetranucleotide repeat motifs were designed using Krait. Since the software was linked to Primer3 (Untergasser et al. 2012), we mined primer pairs directly

from it. We also included compound SSRs as they have few stutter bands. We defined the following parameters for designing primers: product size range, 85–350 bp; maximum mispriming, 12.00; optimum size, 20 bp (minimum 18, maximum 25); optimum temperature, 60°C (minimum, 55°C; maximum, 65°C; maximum temperature difference, 5°C); minimum GC content, 35%, maximum GC content, 65%; maximum selfing, 5 overall and 3 at the ends; and maximum consecutive base repeats, 4 (see Wordley et al. 2011). Other parameters were set as default for the run.

We validated the primers using the *in silico* PCR tool in UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgPcr) against the American alligator genome (Aug 2012; allMis0.2/allMis1 Assembly), as the gharial genome was not accessible in the browser. The default parameters were max. product size, 400; min. good match, 15; and min. perfect match, 15. We excluded primer pairs that gave products of size greater than 400 bp or multiple products. Finally, we exported the annealing temperatures and product size data.

2.3. Wet lab analysis

2.3.1. Screening of mined markers

The final panel of selected SSR markers, after *in silico* PCR, were standardized for their annealing temperatures. Gradient PCRs were performed in 10 µl reaction volume containing 1 µl of 10× buffer (TaKaRa *Ex Taq* hot start version, TaKaRa™), 0.5 µM of labeled forward primer, 0.5 µM of unlabeled reverse primer, 1.0 µl of dNTPs, 2 U *Taq* enzyme (TaKaRa *Ex Taq* hot start version, TaKaRa™), and 1 µl of template DNA (50 ng). PCR reactions were performed in a Mastercycler EP Gradient S (Eppendorf) thermocycler using the microsatellite markers that were selected. The following steps were used in PCR to standardize in annealing temperature: initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 30 s, annealing temperature was set in the range of 55–65°C for 30 s, 72°C for 45 s, followed by a final extension of 72°C for 10 min. We ran the PCR product on a 1.8% agarose gel.

2.3.1. Genotyping

DNA samples (n = 93) were PCR-amplified with selected SSR markers and standardized. We performed PCRs with a 10 µl reaction volume. The PCR products were genotyped in an ABI 3730 Genetic Analyzer using GeneScan 500 LIZ™ size standard (Applied Biosystems). Alleles were scored using GeneMapper software v.5.0 (Applied Biosystems). For each sample, triplicate singleplex PCRs (at each locus) were performed to obtain accurate genotypes.

2.4. Population genetics

2.4.1. Genetic diversity

The number of alleles per locus (k), polymorphic information content (PIC), and probability of identity for individuals and siblings for each locus were determined using CERVUS v.3.0.7 (Kalinowski et al. 2007). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were estimated using GenePop software v.4.7.5 (Rousset 2008), with p-values corrected with the sequential Bonferroni method (Rice 1989). Micro-Checker v.2.2.3 (Van Oosterhout et al. 2004) detected dropouts, null alleles, and errors due to stuttering. Observed (H_o) and expected heterozygosity (H_e) were determined with Arlequin v.3.5 (Excoffier et al. 2005). The inbreeding coefficient (F) was calculated using GenePop v.4.7.5 (Rousset 2008). For all tests, Markov chain parameters were run at 10 000 dememorizations, 100 batches, and 5000 iterations per batch (Weir & Cockerham 1984).

2.4.2. Genetic bottleneck

The presence of a genetic bottleneck event was tested by 2 methods: (1) calculating the Garza-Williamson index (M ratio) in Arlequin v.3.5 (Garza & Williamson 2001, Excoffier et al. 2005) and (2) the heterozygosity excess test (HET) using BOTTLENECK v.1.2.02 (Piry et al. 1999). The statistical power of both tests increases with sample size and the markers used (Peery et al. 2012). An M ratio can efficiently detect ancient ($\geqslant$50 generations) and moderate to severe bottlenecks but is not as efficient at detecting recent and weak population declines ($<$10 generations; Girod et al. 2011). A recent bottleneck that might have occurred in an $N_e < 4$ is likely to be detectable by HET (Luikart & Cornuet 1998), using a 1-tailed Wilcoxon test. A stepwise mutation model (SMM) and 2-phase model (TPM) were used to check for a bottleneck, with 10^4 replications to attain sufficient statistical power (Di Rienzo et al. 1994, Luikart & Cornuet 1998, Piry et al. 1999). The TPM was carried out

with 95% single-step mutations and 5% multiple-step mutations (variance of 12), and the simulations were run for 10 000 iterations based on Piry et al. (1999). Genotyping using 10 polymorphic microsatellite markers was used for both the SMM and TPM.

3. RESULTS

3.1. Development of microsatellite markers

3.1.1. Mining of microsatellite markers

We identified a total of 2014 perfect SSRs and 34 compound SSRs. The total and average length of the SSRs were 86 343 and 42.88 bp, respectively. About 30% of the genome had unknown bps that could not be assigned to any of the 4 nucleotides. The relative abundances of perfect and compound SSRs were 15.6 and 0.26 loci Mb^{-1}, respectively. The relative densities of perfect and compound SSRs were 668.63 and 22.22 bp Mb^{-1}, respectively. The perfect SSRs account for 0.05% of the gharial genome, and compound SSRs account for 3.38% of all the SSRs. The gharial genome had the highest number of dinucleotide repeat SSRs (n = 1303) and the lowest number of trinucleotide repeat SSRs (n = 84; Fig. S1 in the Supplement). A total of 131 tetra repeat perfect SSRs were revealed from the gharial genome. Among all the dinucleotide, trinucleotide, and tetranucleotide repeat SSRs, the motifs AC (n = 990), AAT (n = 52), and ATAG (n = 64) were the most abundant (Fig. S2 in the Supplement). In the gharial genome, the repeats in different motifs varied. They were uniformly distributed except for the AC motif, which was represented 134–990 times.

3.1.2. Primer designing and *in silico* PCR

We mined 84 trinucleotide repeats and 131 tetranucleotide repeats, perfect SSR loci, and 34 compound SSR loci from the gharial genome. Due to the stringent specifications for primer design, including flanking length, temperature, and GC content, we were able to design primers for 76 trinucleotide repeats, 108 tetranucleotide repeats, and 27 compound repeats. The *in silico* PCR analysis of perfect microsatellite primer pairs resulted in amplification for 27 of the 184 primer pairs designed. After excluding the primers that gave products with sizes greater than 400 bp and those with multiple products, we arrived at 22 primer pairs (9 and 13 primer pairs for trinucleotide and tetranucleotide repeats) of perfect microsatellite primers for validation with the wet lab amplification. The *in silico* PCR of compound repeats amplified only 3 pairs of primers. These were taken up further for wet lab tests for PCR amplification.

3.1.3. Wet lab screening of mined markers

Out of 25 SSR primer pairs that were screened for validation using wet lab techniques (Table S2 in the Supplement), 3 primer pairs (GMM2, GMM3, and GMM23) failed to amplify and one primer pair (GMM8) resulted in nonspecific bands. Primer pair GMM8 resulted in a >500 bp product and was omitted from PCR standardization. The remainder of the 21 primer pairs were standardized for primer-specific annealing temperatures.

3.2. Population genetics

3.2.1. Genetic diversity

A panel of 15 polymorphic markers was selected for the population genetic analysis (Table 1) from a total of 21 after eliminating the monomorphic markers (n = 1; GMM1), markers with HWE deviations (n = 2; GMM5, GMM7), and those showing linkage disequilibrium (n = 5; GMM5, GMM7, GMM15, GMM19, and GMM24). The mean amplification success rate for 93 gharials using 15 polymorphic loci was 87%. We did not detect significant allele dropouts, null alleles, or errors due to stuttering. The mean (±SE) PIC was 0.44 ± 0.07. The number of alleles observed at each locus ranged from 2 to 8. The mean number of alleles per locus was 3.73 ± 0.61 (Table 1). The cumulative probability of identity of individuals and the probability of identity of siblings using the markers were both less than 0.001 (Table 2). The mean H_o and H_e were 0.51 ± 0.07 and 0.50 ± 0.07, respectively across 15 polymorphic loci (Table 1). F was measured as −0.03 ± 0.03 (Table 1).

3.2.2. Genetic bottleneck

The M ratio (0.28 ± 0.03) was significantly lower than the critical value (M_c = 0.68) for all the loci, indicative of a genetic bottleneck (Table 3). Similarly, the HET for both SMM and TPM modes (p = 0.02 and p = 0.02 respectively) also identified a genetic bottleneck (Table 3).

Table 1. Details of 15 microsatellite loci screened after wet lab validation and genotype analysis from the gharial genome. T_m: annealing temperature; *k*: number of alleles; n: number of samples; PIC: polymorphic information content; H_o: observed heterozygosity; H_e: expected heterozygosity; *F*: inbreeding coefficient; SE: standard error

Locus	Repeat motif	No. of repeats	Forward primer	Reverse primer	T_m (°C)	Product length (bp)	*k*	n	PIC	H_o	H_e	*F*
GMM4	TAT	10	TTG GTG CTT GGA CTC TTT GC	ACA GAA TTT CTT TTT CCT TCC TGC	58	189–192	2	70	0.22	0.29	0.25	−0.16
GMM6	TAA	12	GGT GTC ACT CCA GGG AAA TCC	CTG GGG TTT TGT CAA CTG TGG	56	333–339	2	79	0.35	0.49	0.46	−0.08
GMM9	TAA	16	TCT GGT CAA GGG AAG AAT GGC	CTT GAT GCA AAG AGG AAA GCG	57	199–216	5	82	0.60	0.62	0.67	0.07
GMM10	TTCA	8	TAA CTG GTC CTT GGG GTT CC	CAA AGA TTT GCA GTT CCT TTT AGC	60	275–287	2	69	0.37	0.67	0.49	−0.36
GMM11	ATAA	9	TGT TAA GTG TTT AGT GGC TGA ATC G	TCC ATT TTG CCA AGT CTG CC	56	229–241	2	74	0.35	0.35	0.45	0.22
GMM12	TAGA	13	CTG CCT GTT CCT TTA CTG CC	TCT GGG CTG AGA ACT TCT TGG	56	271–279	3	82	0.50	0.60	0.57	−0.04
GMM13	AAAG	.8	AAA TGG CAA GTT CAG GTC CC	TGC TGC ATC TGT CCC TTG G	58	196–200	2	82	0.35	0.45	0.46	0.01
GMM14	TTTA	8	GCC TGT GCC AAA ATA TAC TTC C	CGG TCC TTC ACA GCA ATT CC	60	273–277	2	87	0.02	0.02	0.02	−0.01
GMM16	GATA	17	CAG ATG GGG CTT AGG AGA AGG	TGA AAA TTG GGT TTG GCT GC	60	259–283	7	90	0.78	0.83	0.81	−0.0
GMM17	ATCT	17	TGG TTT TGT CTA GAT CAT GTT TCC	ACC TAT CAG TTT CAT TTC AAC ACC C	56	319–348	7	85	0.76	0.81	0.79	−0.03
GMM18	TTGT	43	CTG TGG TGA TGG AAG ACT TTG C	GTT TCC CCT TCT CTC TCT CTC C	62	151–155	2	88	0.32	0.43	0.40	−0.08
GMM20	GTGA	20	TAC TGC GGC ATC ATC ATT CC	AGG AGA ATT TGG TGT GTG AAA TGG	57	299–332	8	84	0.78	0.80	0.82	0.02
GMM21	TAGT	10	TTT GTC TTC CCT GGT GCT GC	ACA AAC AAA CAC CCA ACT CTG C	60	320–336	3	85	0.03	0.04	0.04	−0.01
GMM22	TATC	14	AGC TGT TTC TAA GGG GAG CC	GGC AAT AGT TCT GAA AAG GAC ACC	56	259–287	7	82	0.74	0.81	0.78	−0.04
GMM25	AC–AC	84	TGA GCT GGA CAT TAC ACA CCC	ACG ATT CAA TCC TGC AAC CC	62	273–297	2	89	0.36	0.47	0.48	0.02
						Mean	3.73	81.87	0.44	0.51	0.50	−0.03
						SE	0.61	1.66	0.07	0.07	0.07	0.03

Table 2. Comparison of new markers with previously developed ones using 100 same-scute samples from the Chambal River gharial population (adult female: 12; adult male: 4; subadult female: 22; subadult male: 28; juvenile: 27; unidentified: 7). HWE: Hardy-Weinberg equilibrium; LD: linkage disequilibrium; N: number of markers; n: number of samples; *k*: mean number of alleles per marker; H_o: mean observed heterozygosity; PIC: polymorphic information content; PID (sib): combined probability of identity of siblings; (–): data not available

Microsatellite marker used	Markers screened	Monomorphic or not amplified markers	Markers that failed HWE LD test	N	n	*k* (mean)	H_o (mean)	PIC (mean)	PID (sib)
This study (new markers)	25	5	5	15	93[a]	3.37	0.51	0.44	1.9×10^{-4}
This study (old markers)	18	8	2	8	100[a]	3.12	0.41	0.34	2.4×10^{-2}
Sharma et al. (2021)	27	20	–	7	166	3	0.42	–	4.84×10^{-2}

[a]Same samples were used

Table 3. Comparison of results from population genetic studies of the National Chambal Sanctuary gharial population using different panels of microsatellite markers. *F*: inbreeding coefficient; *M* ratio: Garza-Williamson index; HET: heterozygosity excess test; TPM: two-phase model; SMM: stepwise mutation model

Studies (microsatellite markers used)	No. of samples	Mean *F* (±SE)	Mean *M* ratio (±SE)	HET (p-value) TPM	SMM
This study (new markers) (15)	93[a]	−0.03 (±0.03)	0.28 (±0.03)	0.02	0.02
This study (old markers) (8)	100[a]	−0.01 (±0.05)	0.30 (±0.05)	0.37	0.63
Sharma et al. (2021) (7)	166	−0.03 (±0.02)	0.31 (±0.06)	0.18	0.28

[a]Same samples were used

4. DISCUSSION

4.1. Novel gharial specific microsatellite markers

We used a genome-wide search to mine the 21 novel microsatellite markers (e.g. Amavet et al. 2015, Muniz et al. 2019), which is a more efficient approach than using a traditional approach based on enriched genomic libraries (Miles et al. 2009, de Oliveira et al. 2010). Of the 21 markers, 15 were moderately informative (Hildebrand et al. 1992). The mean numbers of markers and alleles were high in comparison with SSR maker-based studies on other crocodylian species (see Table S3 in the Supplement). The gharial genome had more unknown base pairs and less gene annotation compared to American alligator and saltwater crocodile genomes (Green et al. 2014). In the gharial genome, the abundance of SSR loci was low. However, abundant motifs in all categories were similar to those from other reptiles (Adams et al. 2016, Pasquesi et al. 2018, Liu et al. 2019b). Therefore, our approach was useful for gharial and it contrasts with previous studies employing cross-species microsatellite markers (Jogayya et al. 2013), wherein only 8 out of 18 markers were polymorphic (see Sharma et al. 2021) (Table 2).

The new markers that we developed should be useful for assessing the genetic variation of other extant populations of gharial where small numbers of resident gharial survive, such as in India and Nepal. Such investigation would help to establish the overall genetic variation of the species across its distribution in contemporary times. Furthermore, these markers will likely be useful in examinations of historical gharial specimens in museums and private collections dated across the past 2 centuries. This, in turn, would facilitate comparisons of historic levels of genetic diversity when the total estimated distribution-wide population size (based on breeding adults) was several orders of magnitude larger (> 10 000) than at present (< 1000; Lang 2018, Lang et al. 2019). In addition, it would be valuable for the management of conservation genetic diversity to determine the genetic relatedness of captive gharial in zoo collections as well as those destined to be released for reinforcement and reintroduction into wild habitats. Currently, the captive population of gharial in Indian zoos stands at 137 males and 302 females (Indian Zoo Inventory of 2010−2011; Central Zoo Authority, Government of India, Delhi). Many zoos and multiple rearing facilities in India were seeded from the wild by collecting eggs from NCS (Whitaker 2007). To our knowledge, no genetic profiles were done for captive-reared and/or captive-bred gharial prior to releases in any of the reinforcement or reintroduction schemes conducted under 'Project Crocodile' during the 1970s−1990s, or those in recent decades, or those currently ongoing.

4.2. Genetic diversity

The newly developed microsatellite markers were polymorphic and have demonstrated utility for assessing gharial population genetics. Importantly, the new markers increased the statistical power of inferences about the genetic variation in the NCS population under investigation. The mean PIC value and allelic richness were higher than those reported previously for the NCS population (Table 2).

Genetic heterozygosity of the NCS population was low but similar to estimates of genetic heterozygosity reported for other crocodilian species, including *Alligator sinensis* (Zhu et al. 2009), *A. mississippiensis* (Glenn et al. 1998), *Caiman latirostris* (Amavet et al. 2015, 2021), *C. yacare* (Ojeda et al. 2017), *Crocodylus siamensis* (Yu et al. 2011), *C. mindorensis* (Hinlo et al. 2014), *C. moreletii* (Dever et al. 2002, McVay et al. 2008), and *C. palustris* (Aggarwal et al. 2015, Campos et al. 2018) (Table S2). In general, low genetic diversity can be caused by several demographic factors, such as declines in population size and isolation from

conspecifics resulting in bottlenecks (Gibbs 2001, Frankham et al. 2002, England et al. 2003). Populations facing demographic challenges tend to lose their genetic diversity (Frankham et al. 2002, Garner et al. 2005). Long generation times (ca. 25 yr in the case of gharial), population declines, and demographic challenges may also contribute to low genetic diversity (Eckert et al. 2008, Romiguier et al. 2014, Ellegren & Galtier 2016). Despite the low levels of genetic diversity, the inbreeding coefficient, *F*, suggests that the population studied here is in HWE. Values of *F* are influenced by several factors, such as relatedness among individuals, small population size, occurrence of bottlenecks, admixture, and gene flow (DeGiorgio & Rosenberg 2009, Biebach & Keller 2010, Wang 2017). The lack of evidence of inbreeding is somewhat at odds with the strong evidence that the population underwent a recent genetic bottleneck (see Section 4.3). In the absence of genetic evidence to the contrary, the study population now appears to be in recovery, with minimal or no bottleneck aftereffects evident.

4.3. Genetic bottleneck

Our analyses indicate that the population sampled in this study shows evidence of a genetic bottleneck based on 2 independent measures (*M* ratio and 2 models of HET). Ancient bottleneck events and moderate to severe declines in populations are reliably detected by *M* ratio values (Girod et al. 2011). By contrast, HET models are likely to detect heterozygosity excess for a short time, approximately 0.2–4.0 N_e generations, because a new equilibrium between mutation and drift is reached at the new N_e after this short period (Luikart & Cornuet 1998). Furthermore, HET is sensitive to statistical power and the use of >10 microsatellite markers (Piry et al. 1999).

Historical information on changes in gharial population size in the Chambal drainage is fragmentary, but there is strong support for a gradual reduction in numbers during the past century, from at least ~1000 adults late in the 19th century to ~50 adults by the 1970s (Singh 1978, Lang 2018). Subsequently, the Chambal population has been routinely supplemented by reinforcement, particularly during the late 1970s, 1980s, and 1990s. In recent decades, these efforts have been gradually scaled back to minimal levels in the 2020s. At present, reinforcement continues at low levels (<100 juveniles annually) but only with progeny sourced from the Chambal River. Consequently, the recent demographic changes — namely, a reduction of several orders of magnitude with a relatively recent recovery of at least an order of magnitude — are consistent with our genetic assessment that indicates a genetic bottleneck occurred late in the 20th century. There may have been other, earlier bottlenecks, but this determination is not possible without further analyses that would require historic and recent genetic samples from the region as well as range-wide samples.

4.4. Population genetics of Chambal gharials

This population genetic study is based on scute samples collected from 93 individual wild gharials residing in the lower stretches of the NCS, a distance of about 160 km, from Reha to the Yamuna, which is the bottom third of the approximately 425 km length of the NCS currently inhabited by gharials (Fig. 1). This river segment serves as prime habitat for an estimated 1000 gharials, based on annual direct counts of adults and nests (Gharial Ecology Project 2018). This component represents about half of the total gharial population in the entire Chambal River system. The estimated number of breeding adults in the river segment under study was ~300 females and ~75 males, based on repeated direct counts of adults and the annual number of nests detected. Consequently, our sample represented only a subset of the entire Chambal gharial population and did not include the top two-thirds of the NCS inhabited by an additional 1000 gharials, which included at least ~200 adult females and ~75 males based on repeated direct counts of adults and the annual number of nests detected (Gharial Ecology Project 2018).

At present, it is premature to assume that genetic differentiation is minimal or non-existent between the upstream and downstream segments of the NCS. The sampled segment contains approximately half of the entire NCS population; the other half inhabits the top two-thirds of the NCS where we did not sample. Long-distance movements upstream by subadults tagged downstream in our study area have recently been documented. At least 2 of these individuals were recaptured in upstream locations 4–8 yr later (Gharial Ecology Project 2022). Despite such infrequent events, it has been estimated that migration by 1–10 individuals between putative subpopulations would provide enough gene flow to prevent local adaptation due to isolation (Mills & Allendorf 1996). In addition, extensive seasonal movements of animals tagged in the lower third of the NCS indicate that a significant number of them are breeding and nesting in the mid-

dle third of the NCS (Gharial Ecology Project 2022). These seasonal long-distance movements and occasional shifts in residency from downstream to upstream or vice versa suggest that the entire Chambal gharial population is likely to show little, if any such, genetic differentiation, i.e. the NCS gharial population may be panmictic. If so, then it would constitute a single management unit as opposed to further subdivision.

Additional genetic characterization of the gharials that are resident in upstream stretches of the Chambal is warranted to provide a full picture of the genetic relatedness of the NCS gharial population and should be valuable from a management perspective. In the interim, to ensure the conservation of the Chambal gharial, we suggest that it is necessary to maintain river connectivity through the maintenance of minimum flow levels and protection of intact riverine habitats, such as high sandbanks for nesting and sandbars and mid-river islands for basking.

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