Anthropogenic restocking of gharial individuals prevents genetic isolation of gharial population in Girwa River, India by geographic barriers imposed by a barrage

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Abstract

Gharial (*Gavialis gangeticus*) is a critically endangered fresh water crocodile endemic to the Indian subcontinent. The species has suffered > 95% decline in population and habitat size. A small population of gharials comprising of 50 breeding adults is resident in a 20 Km stretch of the River Girwa in Katerniaghat wildlife sanctuary, India. Gharials in this 20 Km stretch have been genetically isolated since 1976 by a barrage that functions as a barrier to gene flow.

A captive rear and release program has been initiated since 1979 under Project Crocodile for restocking declining wild gharial populations. Thousands of gharial eggs were collected from gharial populations at Girwa and Chambal Rivers. Hatchlings from the collected eggs were captive reared at a common location in Kukrail Gharial Centre, India and released back to multiple gharial populations including the isolated population at Girwa. This restocking programme was not preceded by a genetic screening of captive animals or wild populations to identify genetic diversity and genetic structure of both captive and wild animals.

In this study we investigate whether release of captive reared gharials into the resident population at Girwa River has prevented genetic isolation caused due to barriers imposed by the barrage. Using a combination of empirical analysis using microsatellite markers and a systematic review of data from previous workers on molecular characterization of gharial populations, we analysed genetic differentiation in gharial populations at Girwa and Chambal.

We found similar genetic variability in gharial populations of Girwa and Chambal. There was low inter population genetic differentiation and evidences of genetic migration between the two populations. Our findings indicate that anthropogenic intervention via release of captive animals has compensated for the genetic isolation in Girwa population caused by the barrage.

Introduction

Crocodiles are keystone species in freshwater ecosystems[1]. They face threats from extensive habitat loss and fragmentation, exploitation for skin and meat, resulting in reduced population sizes, altered genetic structure of populations and reduced distribution ranges[2–6]. Population recovery has been achieved for threatened crocodilian species around the world by restocking through captive rear and release programs[7–10].

Project Crocodile, initiated in 1975–76 to recover depleting populations of crocodiles in India is an example of successful restocking of depleting crocodile populations. The Gharial (*Gavialis gangeticus*) is an example of the success of human intervened restocking under Project Crocodile. Gharial is a critically endangered habitat and diet specialist crocodile endemic to the Indian subcontinent. It has suffered > 95% decrease in population and habitat size across its entire distribution range in the Indian subcontinent[5]. Surveys in early 1970's estimated < 200 gharials in the wild. This number has now increased to 650 + mature adult gharials.

According to population surveys made in India in 1973–74 there are two breeding populations of gharial in Girwa and Chambal Rivers respectively[11]. Both populations have been employed as sources for egg collection under Project Crocodile. Hatchlings from eggs collected from these two populations were captive reared at a common site at Kukrail. Since 1979 captive reared gharials from Kukrail have been released back in multiple river systems across India including Girwa and Chambal (Fig. 1). At present, Chambal has the largest gharial population with 500 + mature adults and 400 + nests. This represents ~ 80% of global wild gharial population, followed by Girwa (50 mature adults and 30 + nests)[5]. Captive reared gharials were released in 425 + km stretch of Chambal, whereas, in Girwa, they were released in a small 5 km stretch of the river (Environment, Forest and Climate Change Department, Uttar Pradesh).

Girijapuri barrage built on Girwa in 1976 restricted the resident gharial population to a < 20 km river stretch upstream of the barrage[12]. Two smaller gharial populations located at Chisapani gorge in Karnali (in Nepal, upstream) and in Ghaghra (downstream) are now locally extirpated with no signs of breeding[5,13]. This has made the resident gharial population in Girwa (in Katerniaghat Wildlife Sanctuary) insular, with no dispersal or interactions with other gharial populations due to the geographic barrier formed by the Girijapuri barrage.

Long term assessments of trends in gharial population at Girwa have revealed that there is negligible recruitment in the Girwa gharial population. This is despite external augmentation of gharials since 1979 and yearly addition of wild born hatchlings. Population structure is skewed towards adult animals and the number of small sized gharials (including hatchling and yearlings) remains low[12].

An insular habitat, such as the one formed as a consequence of the Girijapuri barrage on Girwa, mimics a geographic barrier and prevents gene flow in the resident gharial population. However, external release programme of captive reared gharials that has been ongoing since 1979 may have possibly contributed new gene pool to the geographically insular resident population at Girwa. But, as discussed above, long term population trends have shown that the external released animals are not becoming resident in Girwa. Here we analyse the impacts of the geographic isolation caused by the barrage and the external augmentation of the Girwa gharial population on the genetic structure of the gharials at Girwa. We investigate if gene pool of the Girwa population has remained insular or has benefitted from an inflow of genes from other populations. Our findings have important consequences for the success or lack thereof of the decade's long captive rear and release program which is still ongoing. More importantly, does external release even contribute to revival of wild populations in terms of genetic structure?

In this study, we employ microsatellite markers to study the genetic structure of the gharial population in Girwa and Chambal. We further use published and unpublished population and genetic data to determine the effect of external augmentation on gharial populations in Girwa and Chambal Rivers.

Results

Microsatellite genotyping

All six microsatellite markers tested in this study showed amplification with samples from both Girwa and Chambal gharial population. Of the six markers tested, only three were found to be polymorphic (Table 1). G13_1, G13_5, G13_7 and G13_11 were species specific while CpP309 and TGE2 were non-specific. One locus was polymorphic with gharial samples collected at Girwa but was monomorphic with samples collected from Chambal (Table 1).

Consensus genotype, amplification success and error rates

The percent amplification success rates of gharial polymorphic loci were 94.76 ± 1 and 94.09 ± 0.87 for samples collected from Chambal and Girwa respectively. Quality indices for the three polymorphic loci were 0.83 ± 0.03 for Chambal and 0.91 ± 0.12 for Girwa (Table 1). Genotyping scores of the three polymorphic loci is presented in Table 2.

Table 1. Microsatellite markers employed for the study and genetic diversity of Chambal (*n*=10) and Girwa (*n*=30) individuals.

Locus	Number of alleles	Allele Range(bp)	Chambal						Girwa						Reference
			AS (%)	QI	NA	Ho	He	*F*	AS (%)	QI	NA	Ho	He	*F*	
G13_5	2	220–224	93.75	0.9	0.001	–			93.75	0.91	0.0	0.6	0.46	-0.29	14
G13_7	2	264–268	93.75	0.8	0.0	0.6	0.48	-0.25	92.78	0.94	0.20	0.0	0.18	1.0	14
TGE2	2	129–135	96.77	0.8	0.0	0.5	0.37	-0.33	95.74	0.9	0.0	0.83	0.486	-0.71	15
G13_1	1	–													14
G13_11	1	–													14
Cp309	1	–													16

AS=Amplification Success; QI=Quality Index per locus; NA=Null Allele; Ho=Observed heterozygosity; He=Expected heterozygosity; *F*=Inbreeding Coefficient; – = monomorphic; * = not applicable.

Our literature search returned three published studies where microsatellite markers were used to study gharial population in captivity and in wild (Table 3). Of these, one study investigated population genetic structure in captive animals and the other two studied wild gharial populations. In the latter two, one study was restricted to only Chambal gharial population whereas second focussed on both Chambal and Girwa populations. All three studies employed microsatellite markers described by Jogayya et al.[14].

Table 2. Genotyping score for three polymorphic loci used

Sample	Population	G13_7	G13_7	G13_5	G13_5	TGE2	TGE2
K14	Girwa	264	264	220	224	129	135
K15	Girwa	264	264	220	220	129	135
K16	Girwa	264	264	220	220	129	135
K19	Girwa	264	264	220	220	135	135
K2	Girwa	264	264	220	224	129	135
K20	Girwa	268	268	220	220	129	135
K23	Girwa	264	264	224	224	135	135
K25	Girwa	264	264	220	220	135	135
K27	Girwa	264	264	220	224	135	135
K30	Girwa	268	268	224	224	129	135
K31	Girwa	264	264	220	224	129	135
K34	Girwa	264	264	220	220	129	135
K35	Girwa	268	268	220	220	129	135
K4	Girwa	264	264	220	224	129	135
K40	Girwa	264	264	220	224	129	135
K44	Girwa	264	264	220	224	129	135
K45	Girwa	264	264	220	224	129	135
K46	Girwa	264	264	220	224	129	135
K50	Girwa	264	264	220	224	129	135
K55	Girwa	264	264	220	224	129	135
K56	Girwa	264	264	220	224	129	135
K57	Girwa	264	264	220	224	129	135
K58	Girwa	264	264	220	224	129	135
K62	Girwa	264	264	220	224	129	135
K63	Girwa	264	264	220	220	129	135
K64	Girwa	264	264	220	224	129	135
K68	Girwa	264	264	220	220	129	135
K71	Girwa	264	264	220	224	129	135
K73	Girwa	264	264	220	220	129	135
K8	Girwa	264	264	220	224	135	135
C1	Chambal	264	268	224	224	129	135
C11	Chambal	264	268	224	224	129	135
C12	Chambal	264	268	224	224	129	135
C15	Chambal	268	268	224	224	129	135
C16	Chambal	264	264	224	224	135	135
C3	Chambal	264	268	224	224	135	135
C5	Chambal	264	264	224	224	135	135
C6	Chambal	264	268	224	224	129	135
C7	Chambal	264	268	224	224	135	135
C9	Chambal	264	264	224	224	135	135

Population trends and recruitment in Girwa and Chambal populations (1976–2019)

Gharial numbers have increased in both Girwa and Chambal populations. Gharial numbers in Girwa has increased from 14 in 1976 to 70 in 2020[12]. Numbers of gharial individuals at Chambal have increased from ~ 80 in 1976 to 1859 in 2020 (Table 4). Nest numbers in Girwa have increased from 5 to 36 during the years 1976–2020[12], while in Chambal nest numbers have increased from 12 to 443 during 1976–2018 (Table 4). The gharial population at Chambal has substantial recruitment from all size classes, whereas in Girwa, recruitment of gharials especially smaller size classes (yearlings and hatchlings) has been low (Fig. 2)[12].

Table 3: Available data on molecular assessment of gharial populations using microsatellite markers. CAM: Chorioallantoic membrane

Parameters	Jogayya et al. 2013	Singh et al. 2019	Sharma et al. 2021	This study
Number of samples	32	104	348	40
Sample source	Zoological parks	Chambal	Chambal and Girwa	Chambal and Girwa
Sample type	blood/tissue	tissue	tissue/ CAM	CAM
Wild/captive	captive	wild	wild	wild
Number of markers	18	14	27	6
Monomorphic/ Polymorphic	0/18	5/9	11/7	3/3
Markers failed to amplify	0	0	9	0
Number of alleles	2–8	1–8	2–7	2
He/Ho	0.65/0.92	0.44/0.44	Chambal: 0.41/0.42 Girwa: 0.42/0.42	Chambal: 0.43/0.55 Girwa: 0.38/0.0.48

Table 4. Estimated population and nest counts for the gharial population in Chambal River from 1976–2020. Count ranges are shown for available years. GEP: Gharial ecology Project; MPFD: Madhya Pradesh forest Department. Supplementation data was provided by EFCCD, Uttar Pradesh.

Year	Adult males	Adults (> 3m)	Sub-adults (2-3m)	Juveniles (1-2m)	Yearlings (< 1m)	Total	Nest	Supplementation	Reference
1976–81	6–9	28–30	14–149		11–93	83–174	12	438	17–19
1982–86	6–10	27–49	25–182	132–309	45–70	451–628	28–37	598	20–21
1987–92	13–17	97–109	73–143	362–529	254–312	820–982	45–60	459	21–22
1993–99	21	226–238	509–781		232–542	898–1289	64–81	158	23–24
2003		150	265		99	514–540			25–26
2004–08		158–326	272–445		118–322	552–996	64	514	25,27
2009–13	26–36	281–393	127–155	170–216	129–262	870–948	91		25,26,28,29
2017	72	563	365	217	117	1262	417		30
2018	75	591	462	366	208	1627	443		31
2019		1116	254	315	172	1876			MPFD
2020	90	1341	170	262	86	1859			MPFD

Discussion

Our observations in this study indicate that in spite of the isolation caused by a barrier imposed by the Girijapuri barrage, the genetic differentiation between gharial populations at Girwa and Chambal is low. Moreover, an admixture of gene pool as a consequence of intermixing facilitated by the captive rear and release program could have occurred in the two populations. The gharial populations at Chambal and Girwa had similar sized founder populations and received similar external augmentations. Yet the Chambal population has increased substantially in population size and shows higher recruitment rates in various age classes of gharial compared to Girwa population (Table 4; Fig. 2)[12].

Gharial habitat in Girwa is limited to a 20 Km stretch of the reservoir[12]. While there has been an increase in population size, it remains skewed towards adult animals[12]. Small size classes such as yearlings and hatchlings show negligible recruitment in the resident population[12]. It is believed that the Girijapuri barrage functions as a one way exit for resident gharials of Girwa. Barrage gates are opened twice annually, once for maintenance and second time during monsoons. Gharials that are flushed downstream, especially small sized individuals, are unable to enter and recruit in the population after the gates close. Due to a lack of terrestrial locomotion gharials are unable to bypass the barrage through land, as observed in mugger crocodiles. As a consequence, gharials at Girwa are geographically isolated in terms of genetic exchange with other gharial populations. However, under project Crocodile, common rearing of gharial eggs sourced from Girwa and Chambal have resulted in genetic mixing of the two populations[32]. Evidence of gene migration between the two populations despite no habitat connectivity can only be explained by the intermixing at Kukrail[32]. It also indicates recruitment of some of the released animals in the resident Girwa population and hence the low inter-population differentiation.

All wild populations of gharial have been intensively restocked[33] but there is paucity of genetic studies of extant gharial populations[6]. Jogayya et al.[14] studied the genetic diversity of captive populations of gharials using microsatellite markers with samples collected from zoos in India. However, analyses of genetic diversity in wild populations of gharials using the same microsatellite markers reported lower levels of heterozygosity (Table 3)[32,34]. Jogayya et al.[14] had employed only 32 samples that were sourced from gharials separated temporally by decades and with no recent genetic exchange. Consequently, when the same markers were tested on wild breeding populations in Girwa and Chambal there was reduced heterozygosity number of alleles[32,34]. We observed similar levels of heterozygosity as previously reported[32]. Higher values of observed heterozygosity over expected values points to admixture of isolated populations, which is true in case of Girwa and Chambal. But, marker G13_5 was monomorphic for Chambal samples and marker G13_7 showed a null allele frequency of 20% in Girwa samples. Therefore, our data set (3 markers and 40 samples) may reflect bias and provide insufficient evidence to comment upon the population genetic structure. But, similar levels of heterozygosity were reported for gharials of Chambal by both Sharma et al.[32] and Singh et al.[34]. Further, there was similar heterozygosity, low population differentiation and genetic migration between the Girwa and Chambal populations possibly due to intermixing during captive rearing program[32]. Although Sharma et al.[32] have reported that the non-specific marker TGE2 is monomorphic, our results indicate that it is polymorphic (Table 1).

Founder populations play a key role in population dynamics of resultant future captive and wild populations by influencing the genetics[35–38]. Project Crocodile in India assisted in restocking and recovery of gharial. However, genetic screening of the captive or wild populations were never part of the program. Despite evidences of breeding and increase in population sizes in at least three gharial populations from Girwa, Chambal and Ramganga Rivers respectively, genetic diversity of these populations was largely overlooked. This has been the case for several reintroduction and restocking programs globally[39]. Restocking into depleting populations or new habitats can facilitate establishment or recovery of released species, and possible gene flow, or it can cause sudden imbalance of trophic structure and genetic failure in terms of loss of genetic structure, genetic diversity, loss of genetic adaptations, admixture, hybridization etc[39]. Genetic screening of captive and wild animals has been reported in several crocodile species (*Alligator sinesis*[40]; *C. siamensis*[15,41–42]; *C. moreletii*[43]; *C. porosus*[42]; *C. rhombifer*[44]. This has proven useful in identifying pure bred, genetically diverse and in some case hybrid animals.

Habitat and threats play a key role in population genetics in the wild. Habitat connectivity influences dispersal and movement of crocodiles, thereby affecting the availability of breeding adults and mating opportunities (e.g., gharial populations in Betwa, Ken, Mahanadi, Ramganga Rivers). Populations in limited and saturated habitat may employ compensatory mechanism (adult animals regulating recruitment of young sized animals)[45,46]. Killing of adult breeding crocodiles through illegal hunting or fishing may reduce breeding efforts and genetic inputs in subsequent breeding cycles (e.g., Son River gharial population[5]). Gharial population in Chambal is distributed over a stretch of 450 + km of the river and gharials nest on at least 44 locations[5,31]. In Girwa, the population is restricted to ~ 20 km upstream of barrage and gharials nest at only 3 locations of which two are actively maintained by habitat restoration interventions[47]. Further, Chambal population has 90 + male gharials compared to only 6–8 males in Girwa[5]. However, despite substantial differences in habitat, both populations show low genetic differentiation. This could be an outcome of smaller founder population and intensive restocking from a common rearing centre[32].

A majority of studies focussed on genetic screening of captive crocodile populations globally have revealed low genetic variability and inbreeding[43,48–49]. This is due to employing of smaller founder populations from the wild[48]. Release of genetically related captive animals in the wild further aggravates the issue of inbreeding and low genetic variability[43,49]. Crocodiles have long life spans and breed regularly if circumstances

are favourable. Consequently, the breeding animals in captive programs are often the only source of eggs for a considerably long duration and hence reproducing similar genetic variation in released animals[49]. Eggs collected from restocked wild populations or ranching sites are also genetically similar to the captive populations. Wild populations isolated due to geographical barriers e.g., inaccessible marshes, river basins, can show high inter population differentiation[3,50]. However, release of genetically similar captive animals can homogenize these differentiated populations. These factors make it critical for genetic screening of founder populations and released animals.

Anthropocene has contributed to various positive and negative impacts on wild species of flora and fauna. While on one side, human activity pushes wild species populations towards extinction, on the other through conservation initiatives anthropogenic interventions have recovered several species from near extinction. Often these initiatives are able to recover the population sizes, but not the original population dynamics of the species. This is because even though the population sizes are augmented, the habitat is degraded and is unsuitable to sustain the released animals and under anthropogenic threats. For instance, gharial recovery plan in Nepal is facing the stress of excess number of captive gharials without potential release habitat[8,51]. This is due to captive animals growing beyond viable release age, poor recruitment and breeding in released population, skewed population structure and most importantly lack of uniform monitoring and funding to support the recovery program[51].

Due to limited habitat size and absence of natural gene flow, and genetic mixing during captive release program, gharial population at Girwa may be considered as a genetically similar sub sample of the larger gharial population at Chambal. However, release of captive bred animals can contribute in increasing genetic diversity, decreasing fixation of loci and reducing effects of genetic drift induced differentiation, provided the released animals are screened for their genetic structure[3,52]. Low heterozygosity in crocodiles is attributed to longevity, late maturation and generational overlaps[53]. However, traits such as longevity and delayed sexual maturity can result in retention of moderate genetic diversity in wild populations while masking effects of population decline such as genetic drift and low effective population sizes. Considering the life history traits of crocodiles, long term genetic assessments combined with population data should be prioritized in crocodile conservation. Lack of an initial baseline genetic assessment in case of gharials in India leaves a gap in understanding the effects of genetic mixing via restocking, however a detailed assessment of all the extant captive and wild population could provide answers to this question.

Our observations from this study as well as from previous works suggest that at Girwa anthropocene has indeed facilitated gene flow to an isolated gharial population via external augmentation and overcome genetic isolation imposed by a man-made structure that served as geographic barrier to natural population interaction.

Methods

Sample collection and genetic assessment

For gharials at Girwa, extra embryonic membranes were collected from hatched egg shells in the wild. For gharials at Chambal, extra embryonic membranes were collected from hatched egg shells in captivity at Deori Gharial Centre in Morena. The extracted extra embryonic membranes were washed with 70% alcohol and stored in 95% alcohol until further analyses. Two hatched egg shells were sampled from each clutch (clutch $n = 15$ at Girwa and $n = 5$ at Chambal). A total of 40 extra embryonic membrane samples were collected. No live hatchlings or adult animals were handled during the study.

DNA extraction, PCR and genotyping

Genomic DNA was extracted from extra embryonic membrane using the Wizard® Genomic DNA Purification Kit A1120 (Promega corporation, USA) following manufacturer's instructions. Gharial Microsatellite DNA was amplified using the microsatellite marker listed in Table 1. Six microsatellite markers (four gharial specific and two interspecific) were tested. .

All forward markers were labelled using universal M13 adaptor. A 5 µL PCR reaction mixture contained 0.15 µL primer mix, 0.1 µL Q solution, 1.75 µL PCR master mix, 2.5 µL RNase-free water and 1.5 µL Template DNA. The PCR cycles were one cycle for 95°C for 15 min, 8 cycles (95°C for 30 sec, 57°C for 90 sec, 72°C for 30 sec), 14 cycles (95°C for 30 sec, 57–50°C which is decreased 0.5°C/cycle for 90 sec, 72°C for 30 sec), 12 cycles (95°C for 30 sec, 52°C for 90 sec, 72°C for 30 sec) and final extension at 60°C for 30 mins. Positively amplified products were genotyped using a 3130XL Genetic Analyzer, Applied Biosystems. Initial screening of microsatellite markers was performed on 30 samples.

Population genetic structure and differentiation

Consensus genotype, amplification success and error rates

A consensus genotype for each locus was obtained when at least two scores out of three replications were identical[54]. We estimated the average amplification success as percent positive PCR amplification of total PCR reactions performed. To calculate quality index, each repeat was marked as '1' if the replication score was identical to the consensus genotype and '0' if the genotype did not match the consensus genotype. Quality indices were calculated per locus.

Presence of Null alleles was verified using FreeNA[55]. GenAlEx v6.0[56] was used to determine the summary statistic such as number of alleles per locus, observed heterozygosity, expected heterozygosity.

Due to barriers imposed on experiments that were beyond our control because of statutory lockdowns imposed for the COVID-19 pandemic we were unable to test with additional markers. To overcome this shortcoming we performed a literature search on web databases PubMed, Scopus, Web of Science and Google Scholar for similar studies where microsatellite markers were used to study gharials in captivity or wild. We used combinations of keywords that included gharial, microsatellite marker, genetic diversity, heterozygosity, genetic differentiation. We compared results of the published studies that matched the search criteria with results obtained in this study.

Population trends and recruitment in Girwa and Chambal populations (1976–2019)

To compare the population dynamics and recruitment of various size classes of gharials in Girwa and Chambal populations, we compiled population counts for the years 1976–2020 at both sites (Supplementary table 1). Data on number of gharials released from Kukrail Gharial Centre into Girwa and Chambal populations respectively was obtained from Environment, Forest and Climate Change Department (EFCCD), Uttar Pradesh.

Declarations

Ethics

Live animals were not handled during this study. All the data used and analyzed in the study is either presented in this paper or available on publicly accessible web databases.

Data accessibility

Genotype scores used in the study are included as part of the paper. All other data used in the paper are publicly available.

Authors' contributions

GV, SD, FAK and DK conceived and designed the study. GV and SD performed the experimental work. All authors contributed to draft revisions and approved the final version for publication. DK, FAK, PMD arranged all the necessary permissions and funding for the field work.

Competing interests.

The authors declare no competing interests.

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Figures

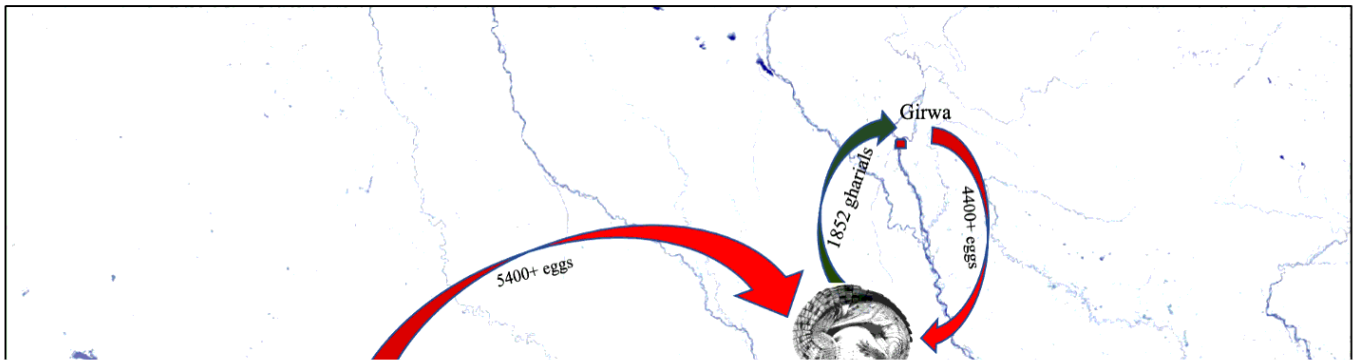

Figure 1

A schematic representation of gharial egg collection and captive gharial release in Girwa and Chambal Rivers. Kukrail gharial centre is the common site where these eggs were incubated and reared in captivity. Map data source: Global surface water data57

Figure 2

Long term gharial population trends in Girwa and Chambal Rivers. The graph represents the estimated total counts for available years including all sized class gharials. The trendline shows a substantial growth in population size in Chambal River relative to Girwa gharial population.

Supplementary Files

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- SupplementaryTable1.docx