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RESEARCH ARTICLE

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Demographic history of the Punjab urial and implications for its management

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Abstract

The Punjab urial (*Ovis vignei punjabiensis*) is endemic to Northern Punjab, Pakistan, and is categorized as vulnerable by the International Union for Conservation of Nature Red List of Threatened Species. The urial population has declined by 30% over the last 3 generations. We used non-invasive fecal samples to identify individuals and estimate population size of Punjab urial in the Kalabagh Game Reserve, Pakistan. We genotyped samples using 12 microsatellite markers to assess genetic variation, population structure, and demographic changes. Microsatellite analysis revealed high levels of genetic variation in urials in terms of expected and observed heterozygosity and allelic diversity. The population structure of the Punjab urial in the Kalabagh Game Reserve, based solely on microsatellite variation using Bayesian clustering, indicated 3 different clusters in the reserve. Results revealed that the urial population may be facing inbreeding pressure because its ancestral effective population size has declined from between 20,000 and 50,000 to ≤1,000 animals today. This reduction has partly occurred because of a bottleneck that occurred about 10,000 years ago. Results also indicate that 1 urial population cluster has the signature of a bottleneck, which may be due to

population isolation. The 3 urial clusters are small and broadly dispersed in a large territory, meaning they could be extirpated without any opportunity for natural re-population through dispersion. The results of our study support a management strategy that encourages maintaining connectivity between urial localities within the Kalabagh Game Reserve, increased diversity so the effective population size may recover from the historical decline, and the use of data generated here as a baseline of urial genetic diversity in the reserve for monitoring diversity over the long term.

KEYWORDS
conservation, genotyping, *Ovis vignei punjabiensis*, urial, wild sheep

The genus *Ovis* comprises a variety of wild and domestic sheep species. While domestic sheep are numerous across most continents, wild sheep have undergone substantial reductions in their population sizes and ranges in recent history. Wild *Ovis* are distributed on mountains of east Eurasia and the western North America (Roberts and Bernhard 1977). The urial (*Ovis vignei*) is a widely dispersed species occurring in Afghanistan, Kashmir, Iran, Kazakhstan, Oman, Pakistan, Turkmenistan, Uzbekistan, and Tajikistan. Because of its population decline in the past 30 years, it is listed as vulnerable (VU) by the International Union for the Conservation of Nature (Michel and Ghoddousi 2020). In Pakistan there are 3 subspecies of urial: Ladakh urial (*O. v. vignei*), occurring in Chitral and Gilgit Baltistan, Punjab urial (*O. v. punjabiensis*), present in the Salt and Kala Chitta ranges in the Punjab, and Baluchistan urial (*O. v. blanfordi*), found in Baluchistan and Sindh (Khan et al. 2015*a*).

The Punjab urial is a key mammalian game species in Pakistan's Salt Ranges, distributed around the Indus River, Jhelum, and the forest belt of the Himalayan foothills. This area is predominantly sub-tropical, dry, semi-evergreen, and scrub forest. The population of Punjab urial occurs as small, scattered groups in Mianwali, Chakwal, Attack, and Khushab districts. The species is herbivorous, grazing on grass and leaves of some trees (Robert 1977, Ayaz et al. 2012), and lives in moderate to arid ecosystems, predominantly grasslands, but can also occur in woodlands and agricultural fields (Roberts 1991). Located about 25 km from the town of Kalabagh, the Kalabagh Game Reserve is one of the biggest reserves (spreading ~36 km^2) of Punjab urial. This reserve was established during the early 1930s on private property of the Nawab of Kalabagh, whose family is still the authority and owners of the reserve. Protection against poachers was vigorously enforced within the reserve where urial have been protected by the Malik family (relatives of the last Nawab) since the 1930s. Livestock grazing within the Game Reserve is strictly prohibited in a core area of 20 km^2 where the greatest urial density occurs, and is limited to a few cattle and sheep in other parts of the reserve where urial also occur in lower numbers. Human access is limited to a few unpaved roads in the lower elevation areas where urial are rarely observed. The Punjab urial is protected under Federal and Provincial laws. Hunting of urial is regulated. Limited trophy hunting is allowed, although illegal hunting of urial has been a significant factor contributing to its decline (Awan et al. 2005). There have been few attempts to characterize population parameters for the species (i.e., population density, population dynamics), and the few genetic efforts to characterize the species have focused on taxonomic questions using mitochondrial DNA markers (Hussain et al. 2015, 2017*b*), or microsatellite markers (Pichler et al. 2017), or have attempted to describe particular loci of interest in the nuclear DNA (e.g., immunity genes; Hussain et al. 2017*a*, 2018). Because of the difficulty of observing Punjab urial in the wild, the few studies conducted focused only on a handful of genetic markers or very few samples because they used DNA sample material that is only collected through handling the animals (e.g., blood). Phylogenetic analyses and comparisons of the similarities in nuclear DNA diversity between Punjab urial and

other *Ovis* species revealed that Punjab urial have unique patterns of DNA variation in their mitochondrial DNA control region, and that the Punjab urial's most recent common ancestor is not the same as that of domestic sheep as previously reported using more slowly evolving mitochondrial DNA genes (Hussain et al. 2015, 2017*a*, *b*; Pichler et al. 2017).

Several population surveys have been conducted to assess Punjab urial population size in the Kalabagh Game Reserve in Northern Punjab, but they relied on traditional field methods such as direct observation of animals or marking individuals, which was labor intensive and impractical for an elusive species. Emerging molecular genetics approaches have helped identify hidden threats to wildlife apart from anthropogenic effects. Populations affected by inbreeding can have an increased frequency of otherwise rare allelic variants, which may have negative effects on fitness (Charleswoth, Willis 2009). Increases in the effect of genetic drift through a decrease in effective population size can result in the loss of genetic variation, thereby reducing the adaptive potential of populations (Charleswoth, Willis 2009). Determining this genetic depletion can only be revealed by using molecular genetics approaches. A combination of population genetics, phylogenetics and other techniques helps unveil the demographic history and present status of the species, and may contribute to future prognoses of population viability and the development of conservation management recommendations (O'Brien 1994, Adams et al. 2019).

We conducted a large-scale analysis of genetic variation of Punjab urial using non-invasive fecal samples from the Kalabagh Game Reserve with a goal of characterizing the genetic variation and historical processes that led to the establishment of the current genetic variation. This goal was achieved through 3 objectives: to quantify genetic variation of the Punjab urial population in the Kalabagh Game Reserve, to evaluate the presence of inbreeding, and to model the demographic history and genetic structure of this wild animal.

STUDY AREA

The Kalabagh Game Reserve is one of the biggest reserves of Punjab urial (~36 km^2) and it is located southeast of the town of Kalabagh in District Mianwali, Punjab, Pakistan, spreading across 7 regions: Jaba, Saran, Dheranwali, Rodhan, Drai, Sokan, and Harnikalan. This reserve was established during the early 1930s on private property of the Nawab of Kalabagh whose family is still the authority and owners of the reserve. Protection against poachers was vigorously enforced within the reserve where urial have been protected by the Malik family (relatives of the last Nawab) since the 1930s. The reserve is on a small massif that is the most westerly part of the Salt Mountains (32°52′N, 71°39′E). The elevation in the Salt Range varies between 250 m to about 1,500 m above the sea level, and urial occur below 1,500 m (Frisina et al. 2007). The climate of the study area was sub-tropical and on average across the last 30 years it had a yearly precipitation of 853 mm. There are 2 distinct rainy seasons: the summer or monsoon rains (April to September) and winter rains (October to March). Temperature ranges between 5.9°C and 38.4°C. Dominant plant species in the area are phulai (*Acacia modesta*), Indian olive (*Olea ferrugenia*), jhal (*Salvadora oleioides*), snatha (*Dodonaea viscose*), honey mesquite (*Prosopis glandulosa*), malabar nut (*Justicia adhatoda*), and aak (*Calotropis procera*), with the sparse shrubs wild jujube (*Zizyphus nummularia*) and bhadrum (*Maytenus royleanus*) occurring through the reserve. The predominant ground cover consists of grasses. Dominant fauna includes the Punjab urial, chinkara (*Gazella bennettii*), chukar (*Alectoris chukar*), see-see (*Ammoperdix griseogularis*), grey partridges (*Perdix perdix*), and black partridges (*Melanoperdix niger*), but also includes prominent carnivores such as Indian wolf (*Canis lupus pallipes*), leopard (*Panthera pardus*), jungle cat (*Felis chaus*), Asiatic jackal (*Canis aureus*), red fox (*Vulpes vulpes*), and yellow-throated marten (*Martes flavigula*; Frisina et al. 2001). Livestock grazing within the Game Reserve is strictly prohibited in a core area of 20 km^2 where the greatest urial density occurs, and is limited to a few cattle and sheep in other parts of the reserve where urial also occur in lower numbers. Human access is limited to a few unpaved roads in the lower elevation areas where urial are rarely observed.

METHODS

Data collection and genotyping

We collected 215 urial sheep fecal samples from 11 different localities (across the 7 regions in the study area) during 4 field surveys of the Reserve (survey locations and buffer scanned areas are shown in Figure 1) between April 2016 and September 2017. We collected fresh samples from the ground by observing urial herds from a distance of ≥0.5 km and then collecting the fecal droppings when the animals left the location. We collected pellets ≥2 m apart of each other to minimize the chance of sampling the same individual twice (this was confirmed with the genetic data). We initially stored samples in sterilized 50-ml centrifuge tubes containing 90% ethanol (Shehzad et al. 2012) for transportation to the laboratory where we further transferred them to a new sterilized 50-ml tube containing 10 ml of silica gel pellets for prolonged storage. From each locality we collected ≥12 samples and out of these, we randomly selected a minimum of 5 pellets per sample for DNA extractions.

We performed DNA extractions in a separate room dedicated to DNA isolation with a starting material of approximately 100 mg of each fecal sample. We used a commercial QiaAmp stool mini kit following the manufacturer's instructions (Qiagen, Germany) and eluted DNA extracts in a volume of 100 µl. We also performed blank extractions in parallel to monitor possible contaminants during extraction process in each batch.

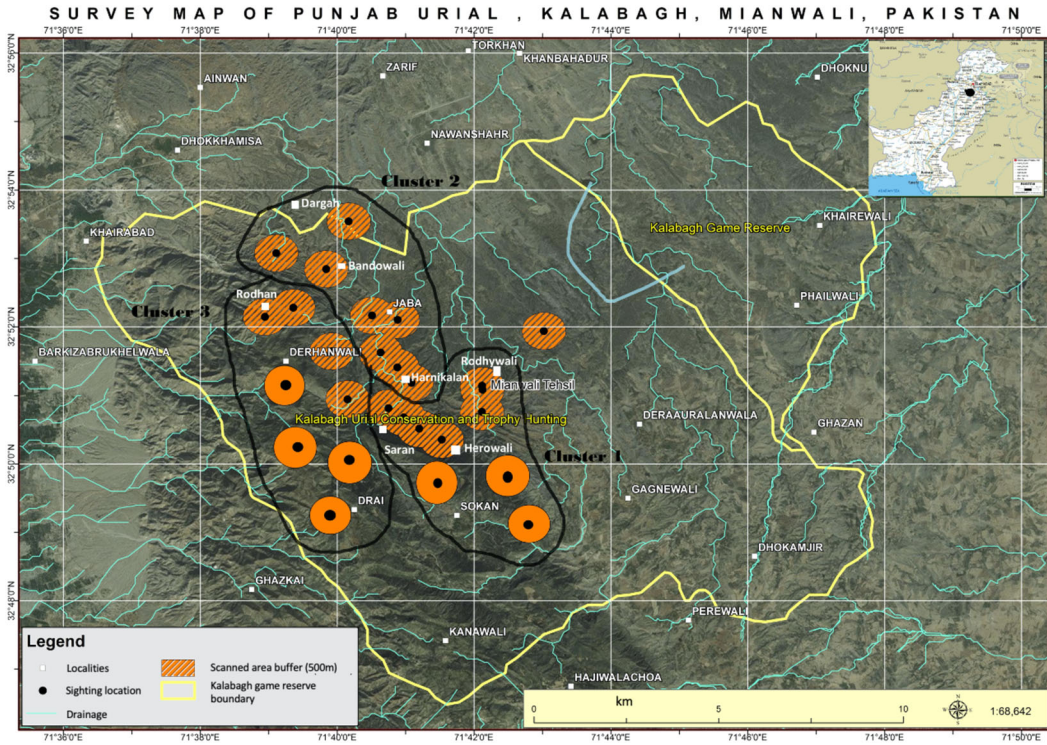

FIGURE 1 Sampling locations of Punjab urials in the Kalabagh Game Reserve, Pakistan, April 2016 and September 2017. The range of the game reserve is shown with a yellow contour line and the areas where the visual searchers located Punjab urials are marked as black spots with a surrounding orange circle indicating the surveyed area. The black colored contour lines correspond to the clusters identified with STRUCTURE. The inset in the figure is the map of Pakistan showing with a black dot the location of the reserve.

We selected a panel of 12 microsatellites (OarJMP29, OarVH72, OarFCB304, OarJMP58, OarFCB226, DYMS1, HuJ616, INRA063, MAF33, MAF70, MAF209, MCM140; Table S1, available in Supporting Information) recommended by the United Nations Food and Agriculture Organization and the International Society for Animal Genetics to amplify in each of sample. We carried out polymerase chain reactions (PCR) in a reaction volume of 20 µl using 0.2 mM of each deoxyribonucleotide triphosphates (dNTPs), 1 X GoTaq® Flexi buffer, 2.5 mM $MgCl_2$, 0.2 µM of each forward (fluorescent labeled with either FAM or HEX) and reverse primer, 1 µl extracted DNA template and 1 U GoTaq® G2 Flexi DNA polymerase, and the following PCR cycling conditions: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 30 seconds at 94°C, 60 seconds at the specific annealing temperature for each primer (Table S1), and 60 seconds at 72°C, ending with a final extension of 10 minutes at 72°C. We visualized amplicons in 0.8% agarose gels for confirmation. For each sample, we pooled together PCR products labeled with different dyes in 4 different sets based on a combination of fluorescent label and PCR product size (Table S1) and sent them for genotyping to the DNA Sequencing and Services, School of Life Sciences, Medical Sciences Institute, University of Dundee, United Kingdom. We determined the allele sizes for each locus for each sample with the Geneious Software (Biomatters, Auckland, New Zealand) and using ET-Rox 500 (GE Biosciences, Pittsburgh, PA, USA) for the size standard. We did not identify duplicated samples when comparing the genotype across all loci between all samples analyzed genetically. We used the software Microchecker (Van Oosterhout et al. 2004) to test for presence of null alleles (i.e., alleles that are present in the biological sample but which the PCR reaction cannot copy), which may derive from random breakdown of DNA before extraction (i.e., due to environmental conditions) and mutational differences between Punjab urials and domestic sheep the species for which the microsatellite primers were designed.

Genetic variation and population structure

We tested Hardy–Weinberg equilibrium (H-W) and linkage disequilibrium (LD) between pairs of microsatellites for each locus in each sampling locality using Genepop version 4.7 (Rousset 2008). Additionally, we computed summary statistics of microsatellite genetic variation (e.g., observed and expected heterozygosity) with MSA version 4.05 (Dieringer and Schlötterer 2003).

We investigated the Punjab urial genetic structure in the Kalabagh Game Reserve using the Bayesian approach implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000). For this purpose, we ran the software STRUCTURE with a burn-in period of 25,000 steps and 100,000 steps for the Markov Chain Monte Carlo (MCMC) data collection phase for a range of number of clusters (K) in the data from 1 to 15. We ran each value of K in triplicate to assess convergence of the MCMC algorithm. We identified the optimal value of K following Evanno et al. (2005). In addition to STRUCTURE, we also performed a principal component analysis using the R package adegenet (Jombart and Ahmed 2011) to determine how samples clustered together using a model free approach. We calculated analysis of molecular variance (AMOVA) with ARLEQUIN version 3.5.2.2 (Excoffier et al. 2005) to determine how genetic variation was partitioned between individuals, localities, or clusters identified by STRUCTURE. We estimated the population pairwise fixation index (Fst) between the clusters identified by STRUCTURE using the software MSA version 4.05.

Demographic changes

We evaluated demographic history of Punjab urial in the Kalabagh Game Reserve using tests that look for population expansions or bottlenecks. For the former we carried out the within-locus *k* and interlocus *g* tests (Reich et al. 1999) using a pre-recorded macro excel sheet (Bilgin 2007). The within-locus *k* test assesses whether the distribution of allele frequencies is centered around a single allele of high frequency (i.e., a leptokurtic

distribution that is typical in a population that has expanded demographically) or whether multiple modes exist in the population as expected in a stable population. The interlocus *g* tests estimates the variance of variances of allele lengths across loci in a population to determine if it is smaller than expected, indicating a population expansion (where most individuals have the same allele) or if it is a large value typical of demographically stable populations that present loci with a wide range of variation in allele sizes. We used the software bottleneck version 1.2.02 (Cornuet and Luikart 1996) to detect recent reductions in effective population size using assumptions of the stepwise mutation model (Kimura and Ohta 1978) and the 2-phased mutation model (Di Rienzo et al. 1994) with default settings.

Lastly, we explicitly modeled changes in the demographic history of Punjab urial using the software MSVAR version 3.1 (Beaumont 1999) to estimate the ancestral effective population size, which at some point may have changed (increased or decreased) until reaching the current effective population size. With MSVAR we estimated the current effective population size (N_0), and the ancestral effective population size (N_t), and estimated the time when the change in effective population (N_t to N_0) size occurred (t). We ran MSVAR 3 times for different priors to test for convergence of the MCMC and to confirm that the posterior estimates of the 3 parameters of interest were not biased by the prior distribution used for the coalescent simulations. We tested 3 different prior models of change in effective population size: a model where the prior assumption of the demographic history was a population expansion, a model where the prior assumption was a stable population in which effective population size does not change, and a scenario where the prior assumption was a bottleneck. We ran MSVAR for 2×10^9 iterations of MCMC algorithm discarding the first 20% of coalescent simulations as burn-in, and assuming an average generation length of 8 years estimated as the average across 5 *Ovis* species (Pacifici et al. 2013). We assessed the convergence of the runs using the Gelman and Rubin's diagnostic (Brooks and Gelman 1998) with the CODA library in R (Plummer et al. 2006). Throughout the MSVAR analyses we assumed an average generation length of 8 years as the average age of first reproduction and accounting for reproductive life span across *Ovis* species.

Genome-wide SNP detection and demographic history

We genotyped 10 Punjab urial samples with Neogene's ovine low-density single nucleotide polymorphisms (SNP) array that has16,560 SNPs. We analyzed data using PLINK version 1.7 (Purcell et al. 2007). Quality control consisted of removing SNPs with ≥10% missing data across all samples. Ascertainment bias is the apparent reduction in genetic variation in a test population (in our case Punjab urial) because the markers that were identified to be polymorphic on the species for which the SNP array was developed (domestic sheep) are not polymorphic on the test population. We minimized ascertainment bias by retaining those SNPs for analyses that had a 10% minimum allele frequency. These filtering steps reduced the dataset to 3,074 SNPs, which we used to estimate heterozygosity and inbreeding coefficients (Fis), and principal components analysis using PLINK and displayed in R (R Core Team 2013). Lastly, we estimated the more recent demographic history of Punjab urial using the software SNeP version 1.1 (Barbato et al. 2015) with default parameters and a maximum distance of 10 million base pairs to account for the low marker density of the array and assuming a generation length of 8 years.

Because of the different time depths facilitated by the microsatellites (recent) and the medium depth provided by the SNP array data, we complemented these analyses with whole genome sequencing for 4 urial samples generated by the NextGen Consortium (Alberto et al. 2018) and analyzed with the multiple sequentially Markovian coalescent (MSMC) to characterize the distant demographic history of the species. Sampling and data generation are described in Alberto et al. (2018). We used the MSMC defaults parameters except for a generation length of 8 years and an average mammalian substitution rate of 1e-8.

RESULTS

We generated 684 genotypes across 12 loci for 57 Punjab urial samples from 11 different localities in the Kalabagh Game Reserve. There was no evidence of null alleles among any of the markers in the 11 localities analyzed (i.e., via Microchecker) indicating that no homozygote genotype resulted from a heterozygote sample failing to produce a PCR product for one of their alleles.

Summary statistics of genetic variation

Overall Punjab urial presented a high genetic diversity in terms of allelic diversity and observed and expected heterozyosity (Table 1). The mean observed number of alleles varied between 1.8 (locality Harnikalan) and 9.0 (locality Dheranwali; Table 1). The mean expected and observed heterozygosity at sub-species level ranged from 0.692 to 0.906 and 0.585 to 1 (Table S1), respectively, with an average observed heterozygosity of 0.85 and average expected heterozygosity of 0.83. The within localities observed heterozygosity ranged from 0.645 (locality Sokan) to 0.937 (locality Rodhywali) and the expected heterozygosity ranged between 0.547 (locality Sokan) and 0.877 (locality Saran; Table 1).

The H-W equilibrium probability test revealed 7 loci significantly deviating from H-W equilibrium (OarJMP29, OarVH72, MAF209, FCB304, HUJ616, DYMS1, and MCM140) when all samples were analyzed as a single group (*P*-values lower than the false discovery rate [FDR] corrected adjusted threshold of 0.016). Because this result could be the outcome of underlying population structure, we carried out the HW equilibrium tests with deficit of heterozygotes as an alternative hypothesis on each locality dataset. None of the localities were out of HW equilibrium because of a deficit of heterozygosity. The test for the alternative hypothesis of excess of heterozygosity, however, resulted in 1 locus (OarJMP58) presenting a significantly larger heterozygosity than

TABLE 1 Summary statistics describing the microsatellite-based estimates of genetic diversity in Punjab urials in Kalabagh Game Reserve, Pakistan, in samples collected between April 2016 and September 2017. We present sample size (*n*), observed heterozygosity (H_o), expected heterozygosity (H_e), average number of alleles per locus (N_A), and inbreeding coefficient (Fis). Values of Fis marked with an asterisk (*) are significant at the 5% *P*-value threshold. NA = Fis value not estimated because the sample size was 1.

Locality	*n*	H_o	H_e	N_A	Fis
Punjab urial	57	0.847	0.831	14.3	
Dheranwali	17	0.918	0.810	9.0	−0.138*
Drai	7	0.869	0.801	6.1	−0.094
Dargah	3	0.833	0.805	3.9	−0.043
Sokan	4	0.645	0.547	3.1	−0.216*
Saran	3	0.861	0.877	4.6	0.039
Jaba	3	0.819	0.858	4.1	−0.063
Rodywali	4	0.937	0.733	3.1	−0.39*
Bandowali	7	0.863	0.848	6.7	−0.018*
Harnikalan	1	0.833	0.833	1.8	NA
Rodhan	5	0.720	0.783	4.3	0.087*
Herowali	3	0.791	0.769	3.3	−0.036*

expected ($P < 0.016$). The LD tests (a correlation in allele frequencies between loci) across all samples resulted in 9 pairwise combinations between loci (13.6% of the number of pairwise combinations) showing LD *P*-values lower than the FDR adjusted threshold of 0.0105 (Table S2, available in Supporting Information). The locality-based LD analysis only found linkage disequilibrium involving 8 loci in the samples of Dheranwali (22% of the loci pairwise comparisons in Dheranwali had *P*-values <0.0105).

Population structure

Bayesian clustering showed the maximum change in likelihood (delta) at K = 3 and K = 10 with no consistent increase in likelihood value between K = 11 and K = 15 (Figure S1, available in Supporting Information). The STRUCTURE bar plots (Figure 2) revealed there were higher clustering solutions (e.g., for K = 6 onwards) that did not increase the resolution of samples grouping with other samples showing a similar membership coefficient to >1 cluster. As such, higher clustering values are likely artefacts, so we focused on the results obtained for the partition solution of K = 3. For the result of K = 3, cluster 1 consists of samples from Sokan, Rodhywali, Herowali and Saran, cluster 2 includes samples from Jaba, Dargah, Bandowali, and Harnikalan, and cluster 3 consists of samples from Dheranwali, Drai, and Rodhan.

Principal component (PC) analysis (Figure 3) was consistent with the STRUCTURE results, in that PC1 separated Sokan from the other populations, whereas PC2 separated individuals from Drai from those in Dargah. The first 2 PCs explained 9.28% and 7.78% of the variance, with the remaining PCs explaining <5.5% of the variance (Figure S2, available in Supporting Information). The low amounts of variance explained by the PCs is consistent with the Punjab urial populations sharing a substantial amount of genetic diversity; 80% of the pairwise Fst values between localities were not significantly different from zero. The remaining pairwise Fst comparisons had an average Fst of 0.1 (Table S3, available in Supporting Information). The AMOVA results for the 11 localities indicated that 5.37% of the variation in the data occurred between localities, while the remaining 94.63% of the variation occurred between individuals within the localities (Table S4, available in Supporting Information). The same analyses between clusters instead of localities identified 8.1% of the variance among clusters while the remaining 91.9% of the variance was between individuals within clusters (Table S5, available in Supporting Information). The pairwise Fst between the 3 pairs of clusters was significant after FDR correction and presented medium-low Fst values, namely 0.049 between cluster 1 and 2, 0.109 between clusters 1 and 3, and 0.132 between clusters 2 and 3.

Demographic changes

The within-locus *k* and interlocus *g* tests showed no significant signature of a recent demographic expansion. The analysis with bottleneck indicated that neither cluster 1 nor 2 show evidence of having been through a bottleneck in their recent history (Wilcoxon test *P*-value > 0.05, and the allele frequency distributions were L-shaped for both clusters). Cluster 3, however, (i.e., urial 3) showed evidence of a bottleneck as indicated by the 1-tailed heterozygosity excess Wilcoxon test under the stepwise mutation model and the 2-phased mutation model (*P*-values 0.017 for both tests). The Wilcoxon test was further supported by a shifted mode (Figure S3, available in Supporting Information).

The MSVAR analysis identified a bottleneck in the distant past of the Punjab urial (Table 2; Figure 4). The independent runs of MSVAR for each population with contrasting prior distributions produced consistent results for the 3 parameters measured (i.e., N_0, N_t, t) and were consistent across independent runs as indicated by all Gelman and Rubin's tests that were lower than the statistic's threshold of 1.1 (Gelman et al. 2013). Five of the 6 populations analyzed consistently indicated a bottleneck that occurred between approximately 7,400 and 8,900 years ago,

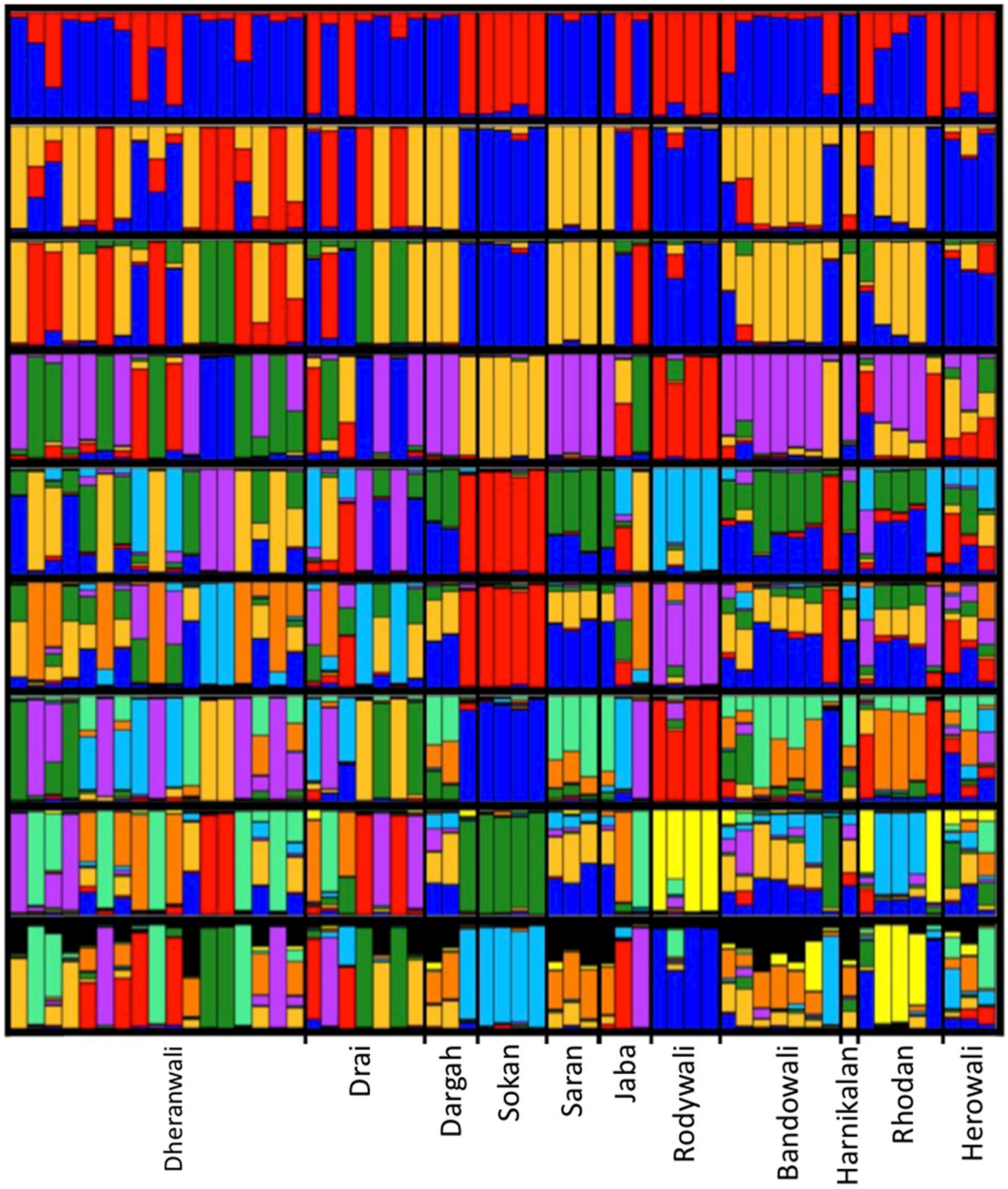

FIGURE 2 Bayesian clustering of Punjab urials in 11 localities in Kalabagh Game Reserve, Pakistan, April 2016 and September 2017. We present clustering results for K = 2 through K = 10. Well defined clusters can be identified between K = 2 and 5; however, from K = 6 onwards STRUCTURE finds additional clusters that only correspond to a fraction of individual admixture proportions.

while the locality of Bandowali urial supports a much more recent bottleneck about 3,090 years ago, although the 95% higher posterior density intervals for the time parameter (245–36,308 years ago) are largely overlapping with those of the 5 other populations (Table 2). The difference in the point estimate of the mode between Bandowali urials and the other populations likely reflects some level of population structure in Bandowali urials that could not be well characterized with the markers and samples available for this study. The bottleneck identified by MSVAR is

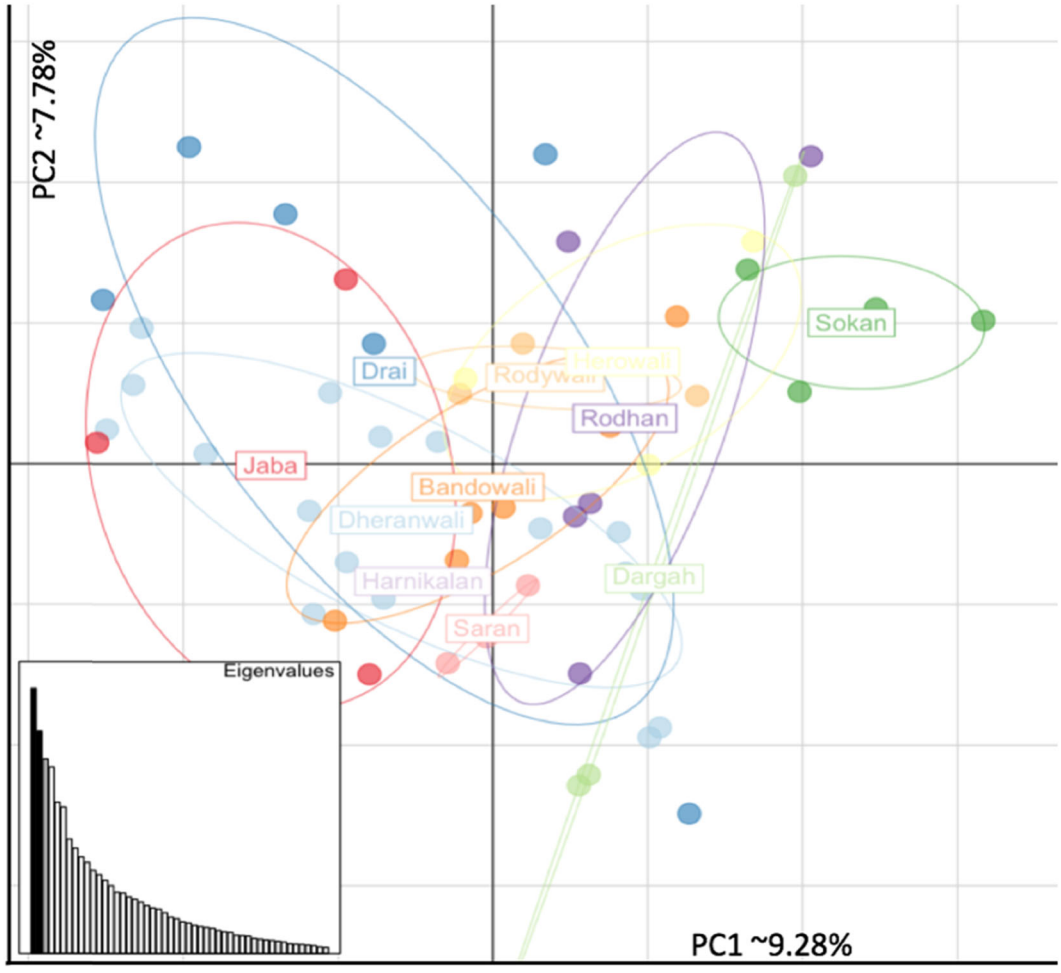

FIGURE 3 Principal component (PC) analysis of Punjab urials in Kalabagh Game Reserve, Pakistan, April 2016 and September 2017. Each population (locality) is in a different color with an ellipse centered at the midpoint of the population's points. The eigenvalues are shown from largest to smallest in the inset.

TABLE 2 Model parameters of the Holocene demographic history of Punjab urials in Kalabagh Game Reserve, Pakistan, in samples collected between April 2016 and September 2017. For each population we provide the mode (M) and the lower (95L) and upper (95U) boundaries of the 95% highest posterior density for the modern effective population (N_0), the ancestral effective population size (N_t), and the time at which the bottleneck occurred (t).

	N_0			N_t			t		
Population	**M**	**95L**	**95U**	**M**	**95L**	**95U**	**M**	**95L**	**95U**
Bandowali	1,096	110	12,023	46,774	10,715	141,254	3,090	245	36,308
Dheranwali	126	288	5,623	57,544	14,454	151,356	7,413	1,622	40,738
Drai	891	251	9,120	37,154	13,490	158,489	7,586	1,259	51,286
Rodhan	2,344	68	6,026	29,512	8,128	114,815	8,913	537	61,660
Rodywali	240	36	2,089	35,481	9,333	144,544	7,762	977	48,978
Sokan	174	8	3,548	22,387	6,026	107,152	7,413	288	38,019

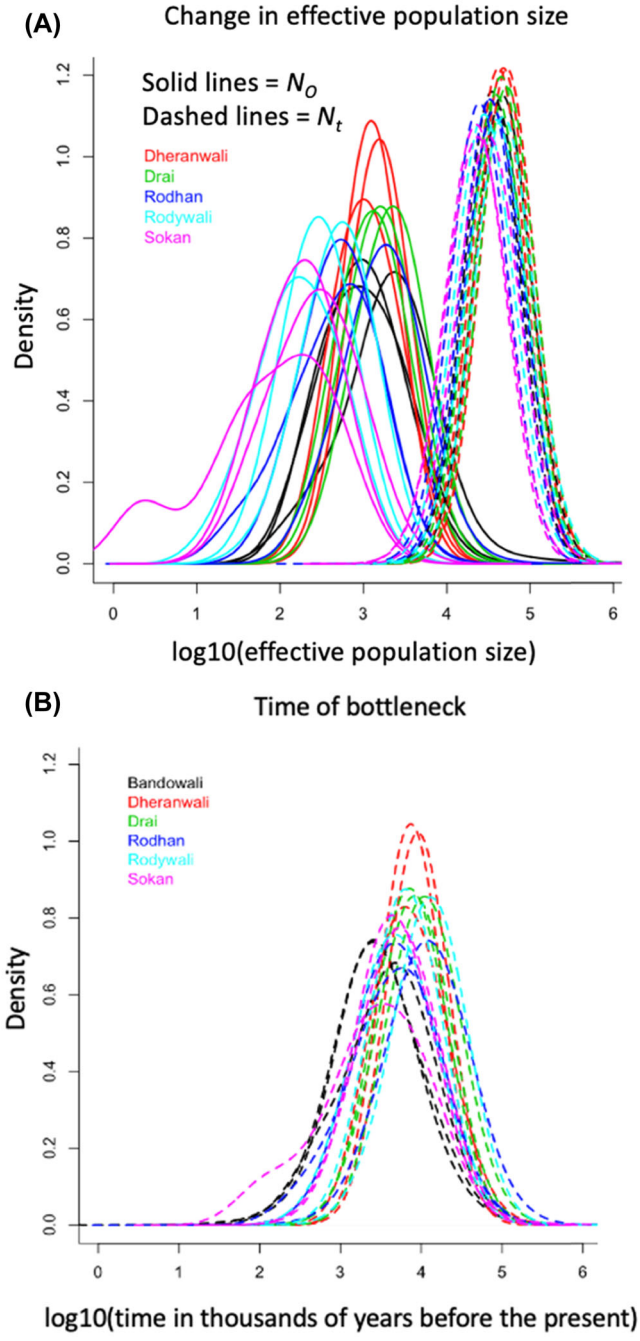

FIGURE 4 Model of Holocene demographic history of Punjab urials in localities in Kalabagh Game Reserve, Pakistan, based on samples collected in April 2016 and September 2017. The posterior distributions estimated for the modern (N_0) and ancestral (N_t) effective population sizes (A), and for the time (t) of the bottleneck (B) are shown. Labels on the x-axis are log10 scale with 1 corresponding to 10^1 until 6 corresponding to 10^6. In A 10^2 is an effective population size of 100, and in B 10^2 is 100 years ago.

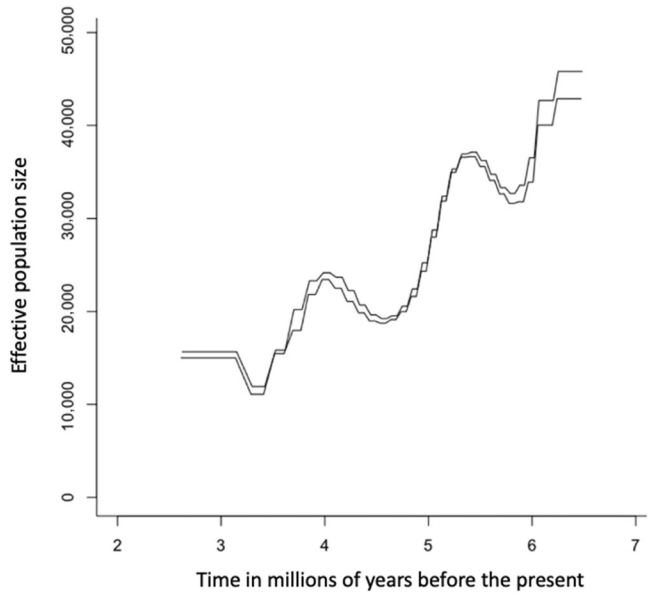

FIGURE 5 Model of ancient demographic history of urials, based on whole genome sequence data from Alberto et al. (2018). We present urial demographic trend over the last 5 million years inferred with multiple sequentially Markovian coalescent. Each of the 2 trajectories shown correspond to a pair of samples analyzed simultaneously.

dramatic with the effective population size reduced from an average of 38,142 N_t to 811 N_O. This result is consistent with the long-term historical effective population size changes in urials as determined with multiple sequentially Markovian coalescent, which shows that the species has experienced multiple population expansions and bottlenecks. After starting with a large effective population size (~50,000), at the onset of the Holocene the species' *Ne* reached half or less than its ancient effective population size (Figure 5) because of multiple previous bottlenecks.

Recent demographic inference

We successfully genotyped 16,560 SNPs in the 10 samples of Punjab urials. After filtering the data to remove SNPs with low call rate and minimum allele frequency of 10%, 3,074 SNPs remained to estimate genetic variation. The average observed heterozygosity was 0.423, with an average expected heterozygosity of 0.371 and an average inbreeding coefficient of −0.141 (Table 3). The SNP-based estimates of genetic variation may seem lower than those of the microsatellites, but this difference arises from various factors. When SNP arrays are designed for a target species or population (in this case domestic sheep) but applied on a different one (in this case Punjab urial), the polymorphic markers identified in the target species may not be variable on the test population, i.e., ascertainment bias (Orozco-terWengel et al. 2015). Furthermore, SNPs in SNP arrays are only biallelic, whereas microsatellites are selected for high polymorphism, and SNP arrays query a significantly larger part of the genome in comparison to microsatellite surveys like ours, and thus are more likely to include parts of the genome that are on average less polymorphic. Nevertheless, both instances of excess of observed heterozygosity observed here are consistent with results for the microsatellites and the negative inbreeding coefficients, which suggest outcrossing of Punjab urial in the Kalabagh Game Reserve with individuals of a genetically different population not captured in this study. The PC1 calculated on these samples separated individuals 108 and 83 from the other 6 samples, whereas PC2 separated individual 65 from the other 9 samples (Table S6). Despite these separations, PC1 and PC2 only

TABLE 3 Summary statistics describing the single nucleotide polymorphism (SNP) array-based estimates of genetic diversity in Punjab urials in Kalabagh Game Reserve, Pakistan, in samples collected between April 2016 and September 2017. We present the observed heterozygosity (H_o), expected heterozygosity (H_e), and the inbreeding coefficient (Fis).

Individual	H_o	H_e	Fis
65	0.379	0.372	−0.020
K18	0.311	0.368	0.154
K19	0.475	0.371	−0.280
K27	0.467	0.371	−0.258
K20	0.548	0.372	−0.472
54	0.508	0.372	−0.366
K30	0.475	0.372	−0.278
108	0.410	0.371	−0.107
K62	0.313	0.369	0.151
83	0.347	0.372	0.068

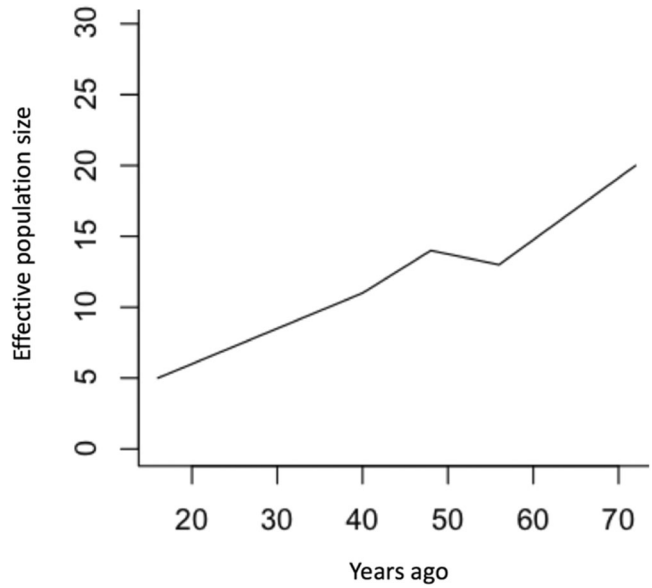

FIGURE 6 Model of the demographic history over the last 100 years in Punjab urials in Kalabagh Game Reserve, Pakistan, based on samples collected in April 2016 and September 2017. The trend in effective population size across the last 70 years was inferred from the distribution of linkage disequilibrium between single nucleotide polymorphisms (SNPs).

account for 3.27% and 1.69% of the variance in the data, respectively (Table S6 and Figure S4, available in Supporting Information). Despite presenting relatively high heterozygosity, the very recent demographic history inferred with SNeP indicates a substantial decrease in *Ne* in the game reserve reaching as few as 5 in the last half century (Figure 6).

DISCUSSION

We evaluated the genetic diversity and demography of Punjab urial in the Kalabagh Game Reserve, using 12 sets of microsatellite markers suitable to extricate the nuclear diversity of this mammal. Microsatellite analysis revealed a high level of genetic variation in urials in terms of observed and expected heterozygosity and allelic diversity (Tapio et al. 2005, Tapio et al. 2010, Pichler et al. 2017, Kovács et al. 2019). Microsatellite heterozygosity calculated for Punjab urial confirmed the observation of a previous study (Pichler et al. 2017) that indicated Punjab urial have high genetic diversity. The Fis values calculated for the localities Dheranwali, Sokan, and Rodywali were the lowest of all areas, probably because these populations have received migrants. The Fis values for Bandowali and Herowali were also negative, and samples from Rhodan indicated some amount of inbreeding (as indicated by the positive Fis). The remaining localities presented Fis values within the 95% bootstrap confidence intervals of this parameter. Nevertheless, all values were very low, suggesting that inbreeding may not play a major role in the demographic history of the reserve's Punjab urials. From a conservation point of view, maintenance of processes that enable animals' genetic exchange between the localities (or between the clusters) should be secured to minimize the chances of inbreeding depression. While the result of SNP array showed lower heterozygosity in terms of observed heterozygosity, the results are similar to those previously observed for mouflon (*Ovis orientalis*) populations (Barbato et al. 2017). The Fis indicates that high rates of inbreeding are not occurring in the population, and on the contrary, there are animals with negative inbreeding coefficients, suggesting that they are the outcome of migrants coming from outside of the reserve or moving between localities to reproduce. These results provide information for urial management in the reserve, as they demonstrate that this is an open population able to receive immigrants that can contribute to maintaining genetic variation of animals in the reserve.

The population structure of the Punjab urial, based solely on microsatellite variation using Bayesian clustering indicated 3 different clusters of Punjab urial occurring in Kalabagh Game Reserve. The 11 different localities within the reserve showed a clustering pattern in which cluster 3 demonstrated the maximum number of collected samples (~43%) belonging to localities Dheranwali, Herowali, and Hernikalan. Analysis of molecular variance showed little between-cluster variation, while most variation was within clusters. This implies that the populations are not much different from each other, and that most of the differences observed between chromosomes are between individuals within populations. The AMOVA results indicated that most of the variation occurs between individuals within localities (or within clusters for the cluster-based AMOVA) rather than between the localities, indicating that localities share many of the alleles and at similar frequencies. We also observed that the historical effective population size was approximately 20,000–50,000, which has been reduced to ≤1,000 today. This change in effective population size has most likely occurred within the last 10,000 years, probably following the mini ice age that occurred 8,000 years ago (Alley and Ágústsdóttir 2005). Both microsatellites and SNPs indicated bottlenecks in the distant past and recently. The results of bottleneck analysis also indicated that cluster 3 of the urial population has a bottleneck signature, whereas cluster 1 and 2 did not show any bottleneck signature. This may be because the bottleneck software did not pick up the bottleneck signal, as it needs a minimum sample size (5–20 polymorphic loci and 20–30 individuals) to be powerful (Hoban et al. 2013). The MSVAR analyses indicated a bottleneck older than the period in which the software bottleneck could have identified a bottleneck (hence the discrepancy between these results). Furthermore, the variation in the bottleneck time estimate of MSVAR for the different localities is largely noise, as the 95% credibility intervals of the posterior probability distributions of the time parameter of the different localities are overlapped.

Despite presenting a relatively high heterozygosity, the very recent demographic history inferred with SNeP suggested a substantial decrease in *Ne* in the game reserve reaching a value of 5 in the last half century. The Punjab urial observed heterozygosity and *Ne* are only a fraction of that observed in other Asiatic ovines (Kijas et al. 2012). The divergence measured with the microsatellites and the SNPs is likely to differ to some extent, which is expected. The divergence measured with microsatellites was approximately 5%, whereas with the SNP array it was about 3%, which is basically the same very small amount of divergence.

Though several researchers previously evaluated population dynamics of the Punjab urial, they were based upon either anecdotal evidence or using visual surveys (Frisina et al. 2001, Awan 2006). Only a few studies (Pichler et al. 2017; Hussain et al. 2015, 2017*b*, 2018) were conducted using molecular markers, and these used invasive sampling to collect blood as source of DNA. Because urial are quite shy and a fragile species prone to either capture myopathy or deaths of individuals during handling (Bartsch et al. 1977, Breed et al. 2019), we focused on non-invasive fecal sampling for DNA sample collection. Future studies can be designed using the approaches implemented here including the use of genomics methods from non-invasive sampling to avoid mortality of urial from research activities. Although we used a relatively small number of samples in this study, they are a representative sample of the reserve's population. With this sample size, we expected that the entire reserve's Punjab urial genealogy is included with a probability of 98.2% (Hein et al. 2004). Additional studies should be designed to collect samples from urial populations outside of the reserve to capture the sources of migrants that move to the reserve and reproduce and to better characterize the demographic context in which the reserve's urials occur.

The Punjab urial is an animal of traditional special interest in Pakistan. Its populations have declined by 30% over the last 3 generations (Valdez 2008) with the species facing poaching and hunting pressure, which have prevented the population from recovering (Awan et al. 2006, Khan et al. 2015). We have identified hitherto unknown genetic structure and demographic changes of the urial population present in the Kalabagh Game Reserve. Our data indicate that urials are distributed in 3 clusters and cluster 3 shows some signs of inbreeding, which may be because of habitat isolation. In the Kalabagh Game Reserve the 3 clusters are small and widely dispersed in fairly accessible territory. This prevents the natural re-population through dispersal into the game reserve, as hunters can access animals everywhere.

MANAGEMENT IMPLICATIONS

Our results suggest that actions should be taken to focus on the conservation of cluster 3, which includes Dheranwali, Drai, and Rodhan. Although the area is protected, a thorough assessment of the urial population size in the reserve should be carried out to determine whether the patterns of divergence observed with the Fst are recovered by field observations, and to support the mixing between the clusters of Punjab urial in the park to avoid their isolation and development of inbreeding. Similarly, the genetic characterization of the reserve Punjab urial should be extended to complement the data generated here and achieve a complete genetic characterization of the urial population genetic variation that can be used as a baseline for genetic monitoring of population health in the future.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

No animals were handled or disturbed during sampling. All samples were collected when animals had left the study sites.

DATA AVAILABILITY STATEMENT

Data (microsatellite genotypes with allele sizes in base pairs) are provided online as file Supplementary Data.xlsx in MSA format in an excel sheet.

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