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Full Length Article

Evaluation of Microsatellite Markers for Genotyping of Markhor (*Capra falconeri*) Populations

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Abstract

Population management and recovery plans play an integral role in the conservation and preservation of the threatened species which can become more effective by amalgamation of genetic data of concerned species. Conservation genetics is one such approach to evaluate genetic diversity and population structure of a species that can direct management strategy of population. Pakistan harbors wide variety of wild Caprinae among which Markhor is the largest wild goat and probable ancestor of domestic goat. Flare horned Markhor (*Capra falconeri falconeri*) inhabits northern areas of Pakistan which are some of world's highest elevated habitats. It is categorized as Near Threatened Globally and Endangered in Pakistan. Being a valuable trophy for hunters, the inception of trophy hunting has increased its population, but still, these species have disappeared from majority of their historic ranges as a result of low reproduction rates, specialized requirements of habitat, genetic isolation in addition to anthropogenic activities. We utilized microsatellite markers for the first time ever to evaluate their applicability and polymorphism for *Capra falconeri falconeri* and genetically characterized meta-population of markhor inhabiting Gilgit-Baltistan. Our results identified microsatellite markers that can effectively reflect upon population genetics of the nominate subspecies. This study also showed depleted genetic variability in one of the sub-populations which needs to be regarded as a conservation priority. All the other studied sub-populations exhibit overall strong sub-structuring. © 2020 Friends Science Publishers

Keywords: Markhor; Microsatellites; Genetic diversity; Population structuring; *Caprinae*

Introduction

In Pakistan, ungulates are the utmost studied large mammals, especially in terms of their distribution and populace counts. Pakistan inhabits seven wild *Caprinae* species (Ali 2008) among which, markhor (*Capra falconeri*) is the most remarkable wild goat because of its massive physique, thick fur, long corkscrew shaped horns, and heavy neck ruff along with flowing beard. It shows the highest horn variations among all the wild *Capra* species (Valdes 2011). Markhor is found in north of India and Pakistan, north-east of Afghanistan, south of Tajikistan and Uzbekistan and south-west of Turkmenistan. It prefers the elevations of 600 m to 3600 m and inhabits arid forest with vertiginous slants and ridges receiving slight precipitation (Roberts 1969; 1997). Nine sub species of (*Capra falconeri*) have been described so far but only three subspecies are currently recognized namely Kabul markhor (*C. f. megaceros*), Astor markhor (*C. f. falconeri)* and *Bukharan markhor (C. f. heptneri)* (IUCN, 2017). Astor markhor (*C. f. falconeri*) is the flare horned markhor. It has extensive distribution in Pakistan as compared to any other country (Hess *et al*. 1997). It has the privilege to be the national animal of Pakistan. Astor markhor is mainly confined to the tributaries of Indus river, Kunar (Chitral) river, Karakoram, Hindu Kush mountain ranges and the Himalayas (Khan *et al*. 2014).

Caprines has the extraordinary ability to survive in harshest environments of earth, but, these species have disappeared from majority of their historic ranges as a result of low reproduction rates, specialized requirements of habitat, genetic isolation in addition to anthropogenic activities such as habitat loss, over-hunting, opening up of preferred grazing areas and forage competition from domestic livestock (Ali 2008; Arshad *et al*. 2012; Ashraf *et al*. 2014). It is general perception of local people, conservationists and wildlife department GB that the instigation of trophy-hunting programmes has abetted the revival of ungulate species in Gilgit-Baltistan, but no scientific study based on a tested methodology has yet been conducted that would ascertain this supposition. Furthermore, there is contradiction about population data as its population was estimated as high as 1500 heads (WCS, 2012) but in recent study conducted by Khan *et al*. (2014),

its population was estimated as 1071 heads. Its population is classified as Endangered in Pakistan according to the IUCN Red List (Michel and Michel 2015). Convention on International Trade of Endangered Species (CITES) has listed Astor markhor on Appendix II (Rosser *et al*., 2005; Sheikh and Molur 2005) and Pakistan's provincial and territorial laws placed it in Schedule I of the Protected Animals (Arshad *et al*. 2012). The persistence of this species is dubious without the development and implementation of conservation approaches.

Conservation genetics is one such approach to evaluate the population viability through determining the heterozygosity levels and genetic diversity of the populations (DeSalle and Amato 2004; Avise 2008). Genetic characterization of a population is done using different genetic markers. Microsatellite markers are one of the best tools for this purpose. They are the co-dominant nuclear DNA markers with high polymorphism (DeSalle and Amato 2004). Highly conserved flanking regions of microsatellites across the taxa allow the amplification across different species of the same genus or family. Next Generation Sequences (NGS) can be used to isolate microsatellites for a species from a genomic source or marker of a neighbouring species can be used instead of developing a new one (Peakall *et al*. 1998). After isolation, a microsatellite is supposed to be polymorphic, if it has a certain repeat number (Schlötterer 1998). They are one of the best genetic markers (Levý *et al*. 2015) to evaluate gene flow, genetic diversity (Waits *et al*. 2000; Hoshino *et al*. 2012), mapping, paternity testing, population genetics (Goldstein and Schlotterer 1999) and kinship studies (Queller *et al*. 1993).

Although, microsatellites have been utilized for number of Caprinae species (Maudet *et al*. 2001; 2002, 2004; Luikart *et al*. 2006; Kanke 2009), their utility for some taxa is not investigated. We used ten microsatellite markers to assess the genetic diversity and population structuring of Astor markhor (*C. falconeri falconeri*) in the Gilgit-Baltistan, Pakistan which has some of largest pockets of populations, to address some of the conservation issues. Some of these markers has already been used for *C.f. falconeri* (Levý *et al*. 2015) and congeneric species (Vahidi *et al*. 2014). The species has shown exponential population increment in recent years in Pakistan (Khan *et al*. 2014) due to community-based conservation efforts owing to its associated economic incentives in the form of revenue generated from trophy hunting. Although it is legally protected in areas of its distribution, but existence in isolated pockets (Khan *et al*. 2014; IUCN 2018) pose threats to its survival. Biogeographic history of species in similar areas (Johannesson 2010; Yamaguchi and Iwasa 2013) has proved that the reproduction of isolated populations embarks the chances of speciation in the long term (Trewick 2016).

Baseline information on population variability, structure and anthropogenic effects on the subpopulation is important for management strategies, as it can identify populations that are at risk of inbreeding depression, or conversely, those sufficiently differentiated to warrant management as a distinct unit. The present study is, therefore, focused on the Astor markhor (*C. f. falconeri*) in the Gilgit-Baltistan, Pakistan to address its conservational issues by assessing its genetic parameters. In this study, 10 autosomal microsatellite loci were utilized to evaluate the genetic diversity and population structuring among the *C. f. falconeri* populations, which have been used in congeneric previously.

Materials and Methods

Sample collection

Prior information about potential sites of species' occurrence was collected from published literature (Schaller and Khan 1975; Khan *et al*. 2014), during field observations, and by interviewing the staff of Forest, Wildlife and Parks Department, GB, shepherds and local people. Fresh faecal samples were collected randomly from 2013 to 2017 throughout its distribution area to ensure uniform distribution of samples across the sampling site. Hence, total of 46 composite samples were collected among which 2 muscle samples were acquired from trophy hunted individuals. The surface of faecal droppings was removed and each sample was transferred into 50 mL tube containing silica for further desiccation and preservation and was stored at -20°C (Nsubuga *et al*. 2004).

Microsatellite genotyping and analysis

Total genomic DNA was isolated from tissue and faecal samples with the Qiagen Tissue Kit and QIAamp DNA Stool Mini Kit. Ten microsatellite markers were chosen (Table 1) depending upon heterozygosity level and high polymorphic information content (PIC; Vahidi *et al*. 2014; Levý *et al*. 2015). Initially all scat samples were screened by amplifying six microsatellite loci via two PCR runs to ensure high quality samples (Roffler *et al*. 2014). Samples that have produced non-identical results at more than three loci were not analysed further. All the selected microsatellite markers were amplified in five Multiplex PCR systems. Universal tail was added in the sequences of forward primers. Tail was fluorescently labelled with NED, FAM, VIC or PET dyes (Applied Biosystems, Foster City, CA, USA).

PCR reactions were done in a total volume of 8 μL comprising: 1–2.0 μL of 50–100 ng/μL extracted DNA, 1.0 μL of fluorescently labelled primer mix, 0.2 μL from 10 mg/mL bovine serum albumin (BSA), 4.0 μL of Qiagen master mix (mixture of Multiplex PCR buffer, dNTPs and Hot Start Taq DNA polymerase) and 0.8–1.8 μL of HPLC or RNase-free water. Amplifications were made using the cycling profile as: initial denaturation for 4 min at 95°C, in 50 cycles; 30 s at 94°C, 30 s at annealing temperature of primer mix, 60 s at 72°C, 60 min at 72°C for final elongation. Amplicons were analysed in ABI 3130 xL DNA

Table 1: Description of 10 autosomal microsatellite loci used to genotype samples of *C. f. falconeri*

S. No.	Locus ID	Gene	Primer sequence 5'-3' (Forward/Reverse)	Reference
1.	SRCRSP8	Dinucleotide repeat polymorphism	F: TGCGGTCTGGTTCTGATTTCAC R: CCTGCATGAGAAAGTGGATGCTTAG	Bhebhe *et al.* 1994
2.	BMS1494	Dinucleotide repeat polymorphism	F: TCTGGAGCTTGCAAAAGACC R: AATGGATGACTCCTGGATGG	-do-
3.	ILSTS029	Dinucleotide repeat polymorphism	F: TGTTTTGATGGAACACAG R: TGGATTTAGACCAGGGTTGG	-do-
4.	MAF035	Dinucleotide repeat polymorphism	F: AGTTACAAATGCAAGCATCATACCTG R: TCAAGAATTTTGGAGCACAATTCTGG	-do-
5.	OarFCB20	Dinucleotide repeat polymorphism	F: GGAAAACCCCCATATATACCTATAC R: AAATGTGTTTAAGATTCCATACATGTG	Buchanan *et al.* 1992
6.	SRCRSP3	Dinucleotide repeat polymorphism	F: CGGGGATCTGTTCTATGAAC R: TGATTAGCTGGCTGAATGTCC	-do-
7.	ETH10	Dinucleotide repeat polymorphism	F: GTTCAGGACTGGCCCTGCTAACA R: CCTCCAGCCCACTTTCTCTTCTC	Toldo *et al.* 1993
8.	ILSTS011	Dinucleotide repeat polymorphism	F: GCTTGCTACATGGAAAGTGC R: CTAAAATGCAGAGCCCTACC	Brezinsky *et al.* 1993
9.	MAF70	Dinucleotide repeat polymorphism	F: CACGGAGTCACAAAGAGTCAGACC R: GCAGGACTCTACGGGGCCTTTGC	-do-
10.	MCM527	Dinucleotide repeat polymorphism	F: GTCCATTGCCTCAAATCAATTC R: AAACCACTTGACTACTCCCCAA	Hulme *et al.* 1995

Analyzer (Applied Biosystems Inc., Foster City, CA). GeneScan-500 LIZ and GeneMapper v. 4.1 (Applied Biosystems Inc.) were used to calibrate and determine allelic size respectively.

Probability-of-identity values (PID and PID sibs) were calculated in GENALEX 6.41 (Peakall and Smouse 2006; Table 2) to evaluate the ability of markers to recognise each genotype (Mills *et al*. 2000; Waits *et al*. 2001). MICROCHECKER was used to evaluate null alleles (Van Oosterhout *et al*. 2004) with an attuned P value, after Bonferroni correction, conforming to $\alpha = 0.05$ (Rice 1989). False alleles and rates of allelic dropout were assessed by replicating 25% of randomly chosen samples, independently. Replicates were excluded by matching individual profiles.

Genetic diversity was estimated for whole sampled population of *C.f. falconeri* and within each genetic sub-population (Table 3). ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010) was used to assess the mean number of alleles per locus (N_A), allele frequencies, observed (H_O) and expected heterozygosity (H_E). The test for deviations from Hardy-Weinberg Equilibrium and pairwise linkage disequilibrium (Guo and Thompson 1992) was also assessed for all locus sub-population in the same program. Sequential Bonferroni correction was done to adjust the p-values in multiple tests (Rice 1989). To compensate the uneven sampling size from each population, allelic richness (N_{AR}) and private alleles richness (N_{PAR}) were assessed, a rarefaction method was used in the software HP-RARE (Kalinowski 2005). F_{ST} (Weir and Cockerham 1984) implemented in GENEPOP v. 4.1 (Rousset 2008) and R_{ST} (Slatkin 1995) determined in FSTAT v. 2.9.3.2 (Goudet *et al*. 2002) was used to assess the pairwise genetic differentiations among the obtained subpopulations. STRUCTURE v.2.3.3 (Pritchard *et al*. 2000; Falush *et al*. 2003) was used to determine population structure.

Results

A cohort of 46 composite samples was acquired from known habitat locations, covering the entire distribution of Markhor in Gilgit Baltistan (GB). No sample was obtained from captive populations of *C. f. falconeri.* Out of the 46 presumptive markhor individuals, 44 (95.6%) unique and high quality consensus genotypes were obtained for 10 microsatellites. 2 samples failed to produce consensus genotypes at more than three loci and were dropped from further analysis. Neither allelic drop-out or false allele nor any identical genotypes were detected in 10 randomly replicated genotypes. The values of probability of identity (PID and PID $_{sibs}$) was very low: PID= 2.7×10^{-34}, PID $_{sibs}$ = 4.8×10^{-13} confirming that different individuals should not have the similar genotype by coincidence. The allele numbers (N_A) across loci ranged from 11 to 19, the expected and observed heterozygosis varied from $H_E = 0.694$ to 0.875 and $H_O = 0.466$ to 0.814 (Table 2). The mean H_O value (0.647±0.036) is significantly lower than H_E (0.795±0.042) with F_{IS} value significantly higher than zero (0.102; $p < 0.001$). Although, few loci deviated from Hardy Weinberg Equilibrium significantly, but upon analysis of discrete Markhor populations (Table 4), no subpopulations showed significant deviation (Table 3). Mk-5 exhibits the highest number of alleles (N_A), as well as allelic richness (N_{AR}) and private allelic richness (N_{PAR}), followed by Mk-4, Mk-1 and Mk-2 populations. Mk-1 and Mk-2 showed almost identical values of number of alleles (N_A) and private allelic richness (N_{AR}). As the heterozygosity is concerned, no significant heterozygosity excess was exhibited by sub-populations in our study, except sub-population of Mk-3 region which exhibited lower values of genetic diversity as compared to other sub-populations (lower N_A, N_{AR}, N_{PAR}, H_o and H_E; Table 3). The *m*-ratio revealed a significant drop in effective population size of Mk-3 region.

Table 2: Genotyping data at each locus of *C.f. falconeri* sub-populations from 10 microsatellites

Multiplex (T_A)	Locus ID	Label	Allele size	N_A	N_{AR}	H_E	H_O	F_{IS}
M1 (55°C)	BMS1494	Fam	210-250	15	8.636	0.795	0.626	0.197*
	SRCRSP8	Ned	207-249	16	8.965	0.793	0.551	0.213*
M2 (57°C)	OarFCB20	Vic	106-146	13	7.315	0.612	0.586	0.11*
	ETH10	Vic	175-255	11	7.012	0.756	0.649	0.128*
M3 (55°C)	MAF70	Vic	125-155	11	7.108	0.76	0.613	0.241*
	MAF035	Ned	98-120	12	7.437	0.731	0.607	0.136*
	ILSTS011	Fam	210-272	16	8.617	0.803	0.645	0.172*
M4 (55°C)	SRCRSP3	Ned	104-142	13	9.795	0.894	0.731	0.196*
	ILSTS029	Ned	142-176	13	9.507	0.743	0.545	0.201*
M5 (56°C)	MCM527	Fam	116-134	9	5.627	0.717	0.6	0.102*

* Significance of F_{IS} values: $P < 0.001$

Table 3: Genetic variation in the genetic clusters of sub-populations at 10 autosomal microsatellites

Macro groups/ Sub-Population	Sub-Populations	N	N_A (± S. E.)	N_{AR}	N_{PAR}	H_o (± S. E.)	H_E (± S. E.)	F_{IS}	HWE	LE
Mk		44	13.293 (±3.211)	8.32	1.4	0.547 (±0.158)	0.741 (±0.180)	0.213*	30	81
Mk-1		10	9.872 (±2.283)	5.62	0.49	0.618 (±0.171)	0.700 (±0.176)	0.189	3	2
	Mk-1.1	8	8.010 (±2.166)	5.77	0.12	0.644 (±0.177)	0.687 (±0.174)	0.079	2	1
	Mk-1.2	2	6.948 (±2.396)	5.51	0.15	0.616 (±0.186)	0.728 (±0.149)	0.163	1	0
Mk-2		8	9.872 (±2.265)	6.11	0.48	0.584 (±0.184)	0.697 (±0.176)	0.176*	15	2
	Mk-2.1	2	6.957 (±1.717)	5.65	0.1	0.619 (±0.203)	0.676 (±0.168)	0.178*	1	0
	Mk-2.2	6	9.310 (±2.225)	5.58	0.13	0.601 (±0.191)	0.691 (±0.182)	0.161*	10	0
Mk-3		4	6.253 (±2.298)	4.34	0.11	0.566 (±0.174)	0.637 (±0.173)	0.1	5	3
Mk-4		15	9.749 (±3.022)	5.37	0.14	0.636 (±0.180)	0.707 (±0.186)	0.279*	16	38
Mk-5		6	10.599 (±2.890)	6.18	0.54	0.663 (±0.160)	0.751 (±0.157)	0.224*	18	14
	Mk-5.1	4	7.835 (±2.072)	6.24	0.28	0.593 (±0.173)	0.757 (±0.122)	0.169*	7	3
	Mk-5.2	2	5.553 (±1.582)	4.57	0.11	0.61 (±0.212)	0.637 (±0.144)	0.949	0	0

N = sample size; N_A = mean number of alleles per locus; N_{AR} and N_{PAR} = allelic and private allelic richness; H_O, H_E = observed and expected heterozygosity; F_{IS} = inbreeding coefficient (*significant departures from HWE at p <0.001, Bonferroni corrected)

Table 4: List of identified genetic clusters of sub-populations

Macro groups	Sub-Population	Location
Mk-1		Gilgit and Hunza Nagar Population
	Mk-1.1	Gilgit, Danyore, Chilmish das, Naltar, Nilolot, Rabat, Hussain Abad, Sikandarabad
	Mk-1.2	Chalt Nagar, Gulmet Nagar, Chaprot, Rahimabad, Bharti, Hopey, Girche, Dassu (Haramosh),
Mk-2		Astore Population
	Mk-2.1	Sassi, Doian, Harcho, Chilisakam
	Mk-2.2	Sassi, Doian, Harcho, Chilisakam
Mk-3		Gilgit Indus Right Bank Population (Darel, Oshkindass, Singul, Haltar Gich)
Mk-4		Skoyo-Karbathang-Basingo and surroundings Population
Mk-5		Gilgit Indus left bank Population-Right of Nanga Parbat towards Astore
	Mk-5.1	Singal, Gittidas, Thor
	Mk-5.2	Thamrus, Pakura, Dimroi, Halaja, Jangal, Tattu, Shawar, Muthath

These results suggest that some past demographic declines might have affected this population and left visible footprints on its genetics.

Bayesian clustering analyses, to assign individuals into putative populations implemented in STRUCTURE v.2.3.3, basically detected five clear genetic clusters that sharply split sampled Markhor populations. The sub-structuring pattern of Markhor populations was even better revealed by the discretion of the data into major geographic-genetic groups (Table 4; Fig 1): Mk-1 (Gilgit and Hunza-Nagar Population); Mk-2 (Astore population); Mk-3 (Gilgit Indus Right Bank); Mk-4 (Skoyo-Karbathang-Basingo and surroundings) and Mk-5 (Gilgit Indus left bank-Right of Nanga Parbat towards Astore). By investigating further subdivision among the macro groups (Mk-1, Mk-2 and Mk-5), more sub structuring split the three macro populations in six distinct clusters that divided macro area Mk-1 into two distinct smaller groups: Mk-1.1 (Gilgit and Hunza-Nagar Population, Gilglit, Danyore, Chilmish das, Naltar, Nilolot, Rabat, Hussain Abad, Sikandarabad) and Mk-1.2 (Gilgit and Hunza-Nagar Population- Chalt Nagar, Gulmet Nagar, Chaprot, Rahimabad, Bharti, Hopey, Girche, Dassu (Haramosh); the macro population Mk-2 into two subpopulations: Mk-2.1 (Doian and Harcho) and Mk-2.2 (Chiliskam); and the macro population Mk-5 into two sub-clusters: Mk -5.1 (Gilgit Indus left bank- Singal, Gittidas, Thor) and Mk -5.2 (Gilgit Indus left bank-Thamrus, Pakura, Dimroi, Halaja, Jangal, Tattu, Shawar, Muthath) (Table 4; Fig 1).

Discussion

Mountain ungulates, especially surrounding Tibetan plateau, have not been much studied for genetic diversity and population structuring due to probable difficulty to access these areas.

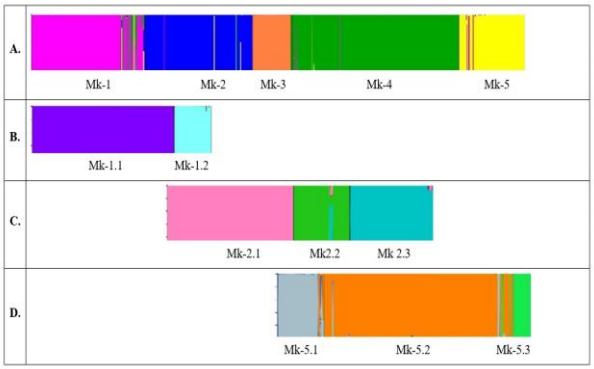

Fig 1: Genetic population structure of A) Markhor population in GB, B-D sub population showing further structuring

However, few primates, bird and lizard species have been explored across the plateau after the revolution in molecular and bioinformatics field which enabled the scientists to answer the questions of conservation concerns (Chakraborty *et al*. 2014; Tan *et al*. 2017). Markhor is legally protected in areas of its distribution. Community-based conservation efforts, such as trophy hunting, led to exponential increment in its population in Pakistan in recent decade (Khan *et al*. 2014; IUCN 2018). However, its survival is threatened due to its existence in isolated pockets (Khan *et al*. 2014; Trewick 2016) as shown by biogeographic history of species in similar areas (Johannesson 2010; Yamaguchi and Iwasa 2013). A mitochondrial DNA study discovered that almost all the captive populations of markhor belong to *C. f. heptneri* species (Hammer and Harper 2008) and are incompetent for reintroduction and recolonization purpose due to introgression. Genetic diversity and structuring of this species had never been explored *via* microsatellites and/or mitochondrial DNA previously, therefore, no data was available for comparison.

Genetic diversity loss may lead to loss of population fitness as reflected in many rare species (Frankham *et al.*, 2003). Moreover, genetic diversity loss may reduce the evolutionary potential of the population in response to alteration in environmental conditions or in species itself as happen in introgressive hybridization. Therefore, the Markhor populations from Mk-3 (Gilgit Indus Right Bank) should be regarded as a conservation preference. Introduction of new breeders in Mk-3 region is of great significance for long-term conservation management of sub-population.

Conclusion

The high genetic diversity and stable population demography of the markhor shows that species had successfully thrived in natural calamities like glacial cycles. Resolving more detailed aspects of the phylo-geographic process, such as meta populations, gene flow and speciation itself, will require the analysis of multiple loci and more number of samples.

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