Microsatellite based genetic characterization and bottleneck analysis of Kari and Madakhlasht sheep breeds from Chitral district of Khyber Pakhtunkhwa, Pakistan

Azmat Ullah¹, Sohail Ahmad², and Muhammad Ibrahim²,*

Objective: Kari sheep show a large variation in body size and gestation length. In this study, we have analyzed the genetic differences between the three subtypes of Kari (based on body size) and another small size breed 'Madakhlasht' inhabiting the Chitral district of Pakistan.

Methods: Animals belonging to small Kari, medium Kari, large Kari, and Madakhlasht sheep breeds were sampled from their breeding region and were characterized genotypically at the DNA level using microsatellite markers. A total of 120 blood samples (30 each) were collected from the four breed types. DNA from these samples were used to amplify 31 ovine specific microsatellite loci through a polymerase chain reaction.

Results: A total of 158 different alleles were detected across the 22 polymorphic loci with an average of 7.18 alleles per locus. Unique alleles were found in all four populations. Significant population differentiation was observed among all the populations. Madakhlasht sheep, phenotypically and geographically more distant from Kari, showed higher population differentiation and lower inbreeding and geneflow with Kari, compared to the values among Kari subtypes. Genetic distance revealed that Madakhlasht was the most distant population among the four populations, whereas, small, medium and large subtypes of Kari sheep were relatively closer to each other. Medium and large Kari sheep were found in the state of mutation drift equilibrium, while small Kari and Madakhlasht showed the presence of bottleneck, indicating a reduction in their population sizes in the recent past.

Conclusion: The results suggest that the three subtypes of Kari possess unique genetic identity and are potentially important for further exploration of their specific characteristics such as productivity and reproduction, and exploitation of their unique values. Madakhlasht needs conservation on a priority basis for future breed improvement programs.

Keywords: Bottleneck Analysis; Genetic Diversity; Kari Sheep; Madakhlasht Sheep; Microsatellite Markers; Molecular Characterization

INTRODUCTION

Livestock plays an important role in the rising economy of Pakistan where sheep are one of the most significant domesticated and abundant species. Out of the whole national livestock holdings, sheep are estimated to be 30.5 million of which Khyber Pukhtunkhwa (KP) province contributes 13% [1]. A total of 33 different sheep breeds have been found in the country, with seven breeds being reported from KP province [2]. However, the actual number of sheep breeds residing in the country is much more. In a recent survey, we have identified 12 different sheep breeds from KP alone (unpublished data).

Sheep are a good source of highly nutritious foodstuff i.e. mutton and milk; contribute valuable raw product (fleece) for the textile industry as well as skin and manure for leather
and agriculture industries [1]. Most of the sheep breeds are well adapted and suitable for their respective environment. In most cases sheep are raised for meat; however, a few breeds such as Damani and Kachi are popular for their milk yield as well [2]. Wool is usually an extra source of revenue to a sheep farmer, wherein some areas fine wool obtained from local breeds is a major product of their sheep flock. The mutton obtained from sheep flock is a good replacement for urea fertilizer, as the price of urea fertilizers is increasing [3].

Kari is a small size thin tail breed that is found only in the Chitral district (The North West of KP province) of Pakistan [3]. The area comprises rugged mountains of Hindukush and Karakorum ranges. Nature has endowed Chitral with 35 narrow and deep valleys. Most of the area is rangeland with scattered vegetation (62%), glaciers and snow (24%), forest (4%), and cultivable land about 3% [4].

The population of Kari sheep is limited in number (probably less than 15,000); however, it is in high demand by the residents for their social and cultural festivities. The consumption of mutton increases by 22% at the household level during the winter, which is very harsh and prolonged in the region [5]. Apart from mutton production, Kari is renowned for the fiber (wool) it produces, which is used by the local cottage industry for making Patti (a woolen cap) and other hand-made woolen fabrics [6].

Kari is a small-sized sheep breed having a thin tail with no definite coat color; however, the white color is predominant. The ears are short, the males having horns while the females are polled. It has a mean gestation length of 110 days and a litter size of 1 to 2, yielding 3.88 lambs/annum. Due to the shorter gestation length, Kari has received much attention recently; however, this unique breed is under the risk of extinction due to the introduction of rams by local shepherds to enhance the wool production and adult weight. It is also at risk due to the movement of Balkhi and Gadak breeds from the Afghanistan side which touches the boundaries of Chitral [3].

The shorter gestation length of Kari sheep makes this breed of great importance for further studying the molecular phenomena underlying this unique character. Usually, the gestation length of sheep ranges from 144 to 151 with an average of 146 days [7]. This unique character is highly variable in Kari sheep ranging from 87 to 153 days. Thus, in some cases, the Kari sheep gestate only for three months. For this reason, the breed has been divided into three categories: i) animals having short (87 to 95 days) gestation length; ii) animals having medium (120 to 123 days) gestation length; and iii) animals having long (151 to 153 days) gestation length. In addition to variation in their gestation length, these different subtypes of Kari sheep also vary in size, such that animals having shorter gestation are smaller in size and animals having longer gestation are larger. Furthermore, the gestation length in Kari sheep has been reported to be influenced by the breeding season and locality of the animals [8].

Several molecular markers are used to detect allele frequencies at different loci among different populations [9]. These markers include simple sequence repeat (SSR), randomly amplified polymorphic DNA, amplified length polymorphism, restriction fragment length polymorphism, single nucleotide polymorphic, and variable number tandem repeat. Microsatellites, also known as SSR, are highly variable; they show a different number of repeated alleles at each locus and differ from each other in the number of repeats [10]. Microsatellites are still the markers of choice for genetic diversity studies in farm animals as well as for parentage analysis, marker-assisted selection, and mapping quantitative trait loci. Microsatellite or DNA markers are preferred over other markers because they are highly polymorphic, co-dominant inheritance, simple in analyzing, and easy to score [11].

The genetic resources of native sheep breeds are at risk of extinction due to extensive crossbreeding [12]. Some of the private shepherds have small flocks and practice crossbreeding, which makes conservation strategies difficult to apply. However, it is essential to define strategies for the conservation of the genetic resources of domestic livestock breeds to preserve their beneficial characteristics. Molecular, phenotypic, genotypic, production system of sheep flocks, existing indigenous knowledge, socioeconomic status, etc. are the basic sources of information that must be pooled before developing preserving methodologies [13].

Molecular characterization is a vital and powerful tool to determine the genetic difference within and among breeds of different livestock species. In this study, we have analyzed genetic diversity in populations belonging to Kari and Madakhlasht sheep breeds from the Chitral District of Pakistan, using FAO recommended ovine specific microsatellite DNA markers [14]. We have evaluated the genetic variability, population structure, and bottleneck analysis of the three subgroups of Kari sheep (small, medium, and large Kari) from upper Chitral and Madakhlasht sheep from lower Chitral.

**MATERIALS AND METHODS**

All the following animal procedures were conducted after a thorough review and approval (99-agr-U-6159) by the Institutional Animal Care and Use Committee of the University of Agriculture Peshawar.

**Animal selection and Blood sampling**

Animals belonging to Kari sheep subtypes (small, medium, and large) and Madakhlasht sheep were randomly chosen from their breeding region (Chitral) based on differences in
their body size and morphology. Per subtype 30 animals were selected for blood sampling, making a total of 120 samples from the four breed types. A total of 3 ml blood sample was collected from each selected individual. Blood was taken from the jugular vein using a disposable syringe and was immediately transferred to the pre-labeled vacutainer. The vacutainers containing blood samples were transferred on ice to the laboratory where these samples were stored at –20°C until DNA isolation.

Isolation of genomic DNA from blood
Genomic DNA was isolated from whole blood samples using standard phenol-chloroform extraction process along with DNA extraction buffer containing sodium dodecyl sulfate (20%), NaCl (400 mM), Tris base (100 mM), ethylenediaminetetraacetic acid (100 mM), and proteinase K (1 mg/mL). The DNA was further used in a polymerase chain reaction to amplify microsatellite regions using specific primers.

Polymerase chain reaction
The polymerase chain reactions were conducted in a 15 μL reaction volume. Each reaction tube comprised 100 ng genomic DNA, 0.25 μM primer (both forward and reverse), 200 μM dNTPs, 1× Taq buffer including KCl, 1.5 mM MgCl₂, and 1 unit of Taq DNA polymerase. The thermal profile was as: an initial denaturation at 94°C, followed by 30 cycles with each cycle consisted of a denaturation step of one minute at 94°C, an annealing step of one minute at primer specific annealing temperature [15], and an extension step of 2 minutes at 72°C. The last cycle was followed by a 10 minutes extension step at 72°C. GeneAmp PCR System 2700 (Applied Biosystem, Grand Island, NY, USA) programmable thermocycler was used for the amplification reactions. A total number of 31 ovine specific microsatellite markers were used individually in the amplification reaction with every individual DNA sample. The polymerase chain reaction (PCR) products obtained after amplification were analyzed on 10% polyacrylamide gel along with the 50 bp DNA ladder.

Data collection
The banding pattern formed by the PCR products on polyacrylamide gels was scored manually, and the size of each band was calculated by plotting its position on the standard curve made of the 50 bp DNA ladder. For each primer and sheep individual, a genotype was assigned based on the band size, homozygosities (giving single band), and heterozygosity (giving double band). The data were further analyzed using statistical software (see below).

Statistical analysis
Variations in the banding pattern of microsatellite markers among the three subtypes of Kari and Madakhlasht sheep were analyzed using different computer software packages. For each population, observed and effective number of alleles, observed and Nei’s unbiased expected heterozygosities, and allele frequencies were computed by using POPGENE software version 1.31 [16]. The Ewens-Watterson test to check the neutrality of the microsatellite loci in each of the four populations was performed at 10,000 simulated samples using POPGENE. A Chi-square test was performed to calculate probability values for Hardy Weinberg equilibrium (HWE) at each locus within each of the four populations. The p-values for HWE of all the loci were then averaged to get the mean p-value of HWE for each population.

Nei’s unbiased genetic distances, identities, and gene flow (Nm) between combinations of the four populations were also calculated using POPGENE. The dendrogram was constructed based on Nei’s genetic distance using the unweighted pair group method with arithmetic mean (UPGMA). The formula by Botstein et al [17] was used for the calculation of polymorphism information content (PIC) values for every locus, in each population, using the allele frequency data generated by POPGENE. The PIC values of all the polymorphic loci within each population were then averaged to get a mean PIC value for each population. Null alleles and their frequencies were calculated using software GENEPOP version 4.3 [18].

To quantify the inbreeding estimates and population substructure, Wright’s fixation indices: \( F_{IS} \) (an indication of intrapopulation genetic divergence), \( F_{IT} \) (between populations inbreeding), and \( F_{ST} \) (interpopulation genetic differentiation) were calculated using FSTAT Version 2.9.3 [19]. Allelic richness (\( A_{ur} \), the measure of allele number with equal frequencies corrected for sample size) was also calculated using FSTAT. Probability values for population inbreeding (\( F_{IT} \)) and differentiation (\( F_{ST} \)) were calculated using the exact G-test in FSTAT based on 1,000 randomizations, assuming random mating within the samples.

The allele frequency data obtained from the POPGENE analysis was used in the BOTTLENECK software version 1.2.02 [20] to test the mutation drift equilibrium (whether the effective population size has been maintained or reduced in the recent past) in the populations. The software computes this effect by taking the allele number and frequencies data, at polymorphic loci, as input. Four statistical tests were performed in BOTTLENECK software namely, sign test, standardized differences test, Wilcoxon sign rank test, and a qualitative test of mode shift. Each of the first three tests calculates loci with heterozygosities excess under the stepwise mutation model (SMM), infinite allele model (IAM), and two-phase model (TPM). The mode shift test distributes the number of alleles observed in high to low-frequency classes.

The proportion of individuals correctly assigned to their
RESULTS

We collected 30 samples from each breed/subtype (see Materials and Methods, Animal selection and blood sampling). However, DNA could not be extracted from some samples, in other samples, none of the given microsatellite markers could be amplified, the final sample count in which at least one of the markers gave results were 24, 26, 28, and 21 (Table 1) for the four subtypes. Resampling could not be done as the animals were in a very remote area (the mountainous region of Chital), where access is difficult.

Genetic diversity among the populations

Intrapopulation genetic variation: The microsatellite markers used showed genotype variations between the four sheep populations (Small Kari, Medium Kari, Large Kari, and Madakhlasht). Of the total 31 markers used, nine did not amplify successfully in any of the sheep populations. The remaining 22 markers were found polymorphic, giving collectively 158 alleles overall loci in all populations. Of the 22 polymorphic loci, some did not amplify or were monomorphic in the individual sheep population. The number of polymorphic loci and the total number of alleles generated by them in each of the four populations are given in Table 1. The number of alleles at each microsatellite marker were counted by the differences in sizes of the PCR products on the polyacrylamide gels. An example of the gel picture of Marker OarFCB226 showing the positions of different alleles based on their sizes are given in Figure 1. The number of alleles for polymorphic loci ranged from 2 (OarFCB193) to 12 (OarJMP58 and INRA63) overall populations. The parameters of genetic variability for each population are presented in Table 1.

The number of polymorphic loci was the least (16) in Madakhlasht sheep, giving a total number of 48 alleles in the population (Table 1). Among the Kari subtypes, one marker (OarFCB193) was monomorphic in small Kari and one (BM8125) in large Kari. Small Kari showed a higher mean observed number of alleles (Na) closely followed by medium and large number of alleles of Kari sheep. The lowest Na was observed in Madakhlasht sheep. In individual populations the Na ranged from 2 (SRCRSP1 and HUJ616) to 8 (OarVH72) in small Kari; 2 (MAF33 and OarFCB193) to 10 (INRA63) in medium Kari; 2 (MAF65 and OarFCB193) to 9 (OarVH72, DYMS1, OarJMP58, and INRA63) in large Kari; and 2 (MAF214, OarVH72, OarFCB304, OarJMP29, MAF65, ILSTS5, and BM1329) to 7 (DYMS1) in Madakhlasht population. The mean effective number of alleles (Ne) was lower than Na in all populations. The A_0 was similar across the three subtypes of Kari and lower in Madakhlasht (Table 1).

The observed heterozygosity (Ho) values were lower than their expected estimates (Wahlund effect) in the medium Kari population; however, in small Kari, large Kari, and Madakhlasht populations Ho exceeded the He. The Wahlund effect (less Ho than He) was observed at a few loci (7, 9, and 1, respectively) in small Kari, large Kari, and Madakhlasht populations. While in the medium Kari population the Wahlund effect was observed at more (12) loci. Within population inbreeding estimates (F_is) were smaller and were not significant in all the four populations. In small Kari and Madakhlasht, the values for F_is were negative. Mean PIC was higher in all sheep populations except Madakhlasht (mean PIC<0.5). Within individual populations, the PIC value was less than 0.25 for one locus (OarFCB193) in large Kari, and one locus (SRCRSP9) in the Madakhlasht population. Averaging the p-values of HWE at the polymorphic loci in each population revealed that overall the populations were in HWE. However, within individual populations, five loci deviated from HWE in small Kari, 14 loci deviated in

| Population     | N  | Np | Nt | Na   | Ne   | A_0 | Ho    | He    | F_is | PIC | HWE p-value |
|----------------|----|----|----|------|------|-----|-------|-------|------|-----|-------------|
| Small Kari     | 24 | 21 | 92 | 4.381| 3.571| 2.790| 0.838 | 0.752 | -0.136| 0.578| 0.275       |
| Medium Kari    | 26 | 22 | 121| 5.500| 3.807| 2.822| 0.742 | 0.757 | 0.006 | 0.628| 0.098       |
| Large Kari     | 28 | 21 | 119| 5.667| 4.447| 2.889| 0.756 | 0.742 | 0.040 | 0.622| 0.152       |
| Madakhlasht    | 21 | 16 | 48 | 3.000| 2.711| 1.743| 0.885 | 0.742 | -0.319| 0.462| 0.499       |

N, sample size; Np, number of polymorphic loci; Nt, total number of alleles; Na, observed number of alleles; Ne, expected number of alleles; A_0, allelic richness; Ho, observed heterozygosity; He, expected heterozygosity; F_is, within-population inbreeding coefficient; PIC, polymorphism information content; HWE, Hardy-Weinberg equilibrium.
medium Kari and 12 loci deviated in large Kari. None of the loci deviated from HWE in the Madakhlasht population.

**Allele sizes and frequencies:** Different alleles, their sizes, and respective frequencies obtained after amplification of microsatellite markers in the four sheep populations are given in Supplementary Table S1. Variation was observed in the frequencies of different alleles in different sheep populations. At a very few loci (3 out of 22) a similar allele was found at higher frequency in all four populations. More similarities were observed in allele frequencies between the three subtypes of Kari compared to among the Kari subtypes and Madakhlasht populations. Small, medium, and large Kari populations showed the same allele at a higher frequency at a total of eight microsatellite markers. At other loci, allele frequencies were variable between different sheep populations. Null alleles were observed at more loci in large Kari (14 loci), followed by medium (12 loci) and small Kari (7 loci). The least number of loci showing the presence of null alleles was observed in Madakhlasht sheep (4 loci) (Supplementary Table S1).

**Shared and unique alleles:** Shared and Unique alleles were found in all the three subtypes of Kari sheep as well as in Madakhlasht sheep (Table 2). Different subtypes of Kari sheep shared more alleles compared to allele sharing between any of the three Kari subtypes and Madakhlasht sheep. In total, nine unique alleles were observed in Small Kari, seven in medium Kari, seven in large Kari, and one in Madakhlasht sheep at different loci. Of the total unique alleles, five in small Kari, two in medium Kari, five in large Kari, and the one in Madakhlasht sheep were relatively more frequent (frequencies exceeded 0.2) than the others (Supplementary Table S1).

**Genetic assignment of individuals to their respective populations:** The percentage of individuals correctly assigned to their respective populations obtained by putting the individual genotype data in GENECLASS software is presented in Table 3. Sample specimens from Large Kari subtypes were assigned at high precision: ranging from 58% to 83% by using different methods. Assigning accuracy of small and medium Kari populations ranged from 50% to 83% and 54% to 72%, respectively. Madakhlasht sheep results were found to be more inconsistent when computed under different methods. The accuracy of the sampled Madakhlasht individuals varied from 25% to 100%.

**Population structure:** To estimate population differences, the fixation indices $F_{IT}$ (total inbreeding estimates) and $F_{ST}$ (a measurement of differentiation) along with $N_M$ (gene flow) among different subtypes of Kari sheep and between Kari and Madakhlasht sheep were calculated using the FSTAT program (Table 4). The mean $F_{IT}$ values were significantly higher among the pairs of all the four populations, having $p<0.05$ under the exact-G test. Mean $F_{IT}$ values were significantly higher
between small and medium Kari and between medium and large Kari populations. The inbreeding estimates ($F_{IT}$) were non-significant between the Kari subtypes and the Madakhlasht population. Similarly, higher gene flow ($N_{M}$) was

### Table 2. Shared and unique alleles in Kari subtypes and Madakhlasht sheep

| Locus         | Number of alleles | SM-ME | SM-LA | ME-LA | SM-MK | ME-MK | LA-MK | SM   | ME   | LA   | MK  |
|---------------|-------------------|-------|-------|-------|-------|-------|-------|------|------|------|-----|
| OarJMP58      | 12                | 4     | 3     | 7     | 2     | 3     | 5     | 2    |      |      |     |
| INRA63        | 12                | 6     | 4     | 8     | 2     | 2     | 2     |      |      |      |     |
| OarVH72       | 11                | 5     | 7     | 5     | 2     | 2     | 1     |      |      |      |     |
| DYM1          | 10                | 6     | 6     | 7     | 4     | 4     | 6     |      |      |      |     |
| OarFCB304     | 8                 | 2     | 2     | 1     | 4     | 1     | 2     | 2    |      |      |     |
| ILSTS11       | 8                 | 2     | 1     | 2     |      |      |      |      |      |      |     |
| OarJMP29      | 8                 | 5     | 5     | 5     | 1     | 2     | 2     |      |      |      |     |
| SRCRSP1       | 8                 | 2     | 1     | 4     | 1     | 2     | 3     |      |      |      |     |
| MAF214        | 7                 | 4     | 3     | 3     | 1     | 2     | 2     |      |      |      |     |
| BM1824        | 7                 | 5     | 4     | 6     |       | 1     | 1     |      |      |      |     |
| MOM527        | 7                 | 2     | 2     | 4     | 3     | 2     | 3     |      |      |      |     |
| MCM140        | 7                 | 4     | 3     | 5     | 2     | 3     | 3     | 1    |      |      |     |
| ILSTS5        | 7                 | 4     | 2     | 4     | 1     | 2     | 2     |      |      |      |     |
| BM1329        | 7                 | 2     | 3     | 4     |       | 1     | 2     |      |      |      |     |
| SRCRSP5       | 7                 | 3     | 2     | 4     | 1     | 1     | 1     |      |      |      |     |
| HJU616        | 6                 | 2     | 2     | 4     | 1     | 2     | 3     |      |      |      |     |
| OarFCB226     | 6                 | 2     | 2     | 5     |       |      |      |      |      |      |     |
| MAF33         | 5                 | 2     | 3     | 2     | 2     | 1     | 3     | 1    |      |      |     |
| SRCRSP9       | 5                 | 3     | 4     | 4     | 2     | 3     | 3     |      |      |      |     |
| BM1825        | 4                 | 3     | 1     | 1     |       |      |      |      |      |      |     |
| MAF65         | 4                 | 4     | 2     | 2     | 2     |      |      |      |      |      |     |
| OarFCB193     | 2                 | 1     | 1     | 2     | 1     | 1     | 1     |      |      |      |     |
| Total         | 158               | 73    | 63    | 91    | 27    | 38    | 47    | 9    | 7    | 7    | 1   |

SM, small Kari; ME, medium Kari; LA, large Kari; MK, Madakhlasht.

### Table 3. The average probability of individuals correctly assigned to their respective populations using different statistical criteria

| Population | Frequency criteria | Bayesian criteria | Distance criteria |
|------------|-------------------|------------------|------------------|
|            |                   | R&M              | B&L              | $D_S$ | $D_M$ | $D_A$ | $D_C$ | $D_{AS}$ |
| Small      | 66.67             | 66.67            | 50.00            | 83.33 | 83.33 | 66.67 | 66.67 | 83.33    |
| Medium     | 63.64             | 54.55            | 72.73            | 72.73 | 72.73 | 72.73 | 72.73 | 54.55    |
| Large      | 83.33             | 83.33            | 75.00            | 75.00 | 66.67 | 83.33 | 83.33 | 58.33    |
| Madakhlasht| 25.00             | 50.00            | 50.00            | 75.00 | 100.00| 50.00 | 50.00 | 75.00    |

R&M, Ranala and Mountain; B&L, Baudouin and Lebrun statistics; $D_S$, Nei’s standard distance; $D_M$, Nei’s minimum distance; $D_A$, Nei’s average distance; $D_C$, chord distance; $D_{AS}$, shared allele distance.

### Table 4. Pairwise genetic differentiation ($F_{ST}$), total inbreeding ($F_{IT}$), and gene flow ($N_{M}$) among the four populations

| Items                  | Kari subtypes | Kari subtypes | Madakhlasht |
|------------------------|---------------|---------------|-------------|
| Population differentiation | Small         | 0.147±0.021*  | 0.149±0.022** | 0.257±0.037** |
|                        | Medium        | 0.105±0.022*  | 0.163±0.023*  | 0.193±0.042*  |
|                        | Large         | 0.015±0.042   | -0.023±0.040  | 0.008±0.067   |
| Total inbreeding       | Small         | 0.03±0.047*   |               |              |
|                        | Medium        | 0.046±0.041** |               |              |
|                        | Large         | -0.023±0.040  |               |              |
| Gene flow              | Small         | 3.779±0.753   | 4.234±0.721   | 2.999±0.845   |
|                        | Medium        | 9.315±2.609   | 3.674±1.034   |              |
|                        | Large         |                | 4.846±1.866   |              |

* $p<0.05$; ** $p<0.01$ under exact-G test assuming random matting within the samples.
recorded among the Kari subtypes, while the values were less between any of the three Kari subtypes and Madakhlasht sheep. The least $N_M$ was observed between small Kari and Madakhlasht populations (Table 4).

The STRUCTURE analysis grouped different populations in inferred clusters (at the given K value) based on the allele frequency data at each locus (Figure 2). At $K = 2$ the small Kari individuals were grouped in a separate cluster, while the rest of the three populations (medium Kari, large Kari, and Madakhlasht) made one large cluster. This differentiation of small Kari from the other three populations persisted at higher K values. At $K = 3$ the Madakhlasht sheep was separated into a distinct group. The group made by medium Kari at $K = 3$ showed some level of heterogeneity among the individuals. At $K = 4$ a high proportion of individuals (99%) belonging to small Kari and Madakhlasht were grouped in separate clusters. Individuals from medium Kari and large Kari populations showed an admixed ancestry, and only 75% and 59% of individuals, respectively, were purely assigned to separate inferred groups of the two populations.

**Genetic distance among populations:** Estimates for genetic distances calculated among the four populations revealed that Madakhlasht was the most distant population among the four (Table 5). Within the sub-populations of Kari, medium and large Kari showed less genetic distance compared to the genetic distance between either of these sub-populations with small Kari. This effect can also be seen in the dendrogram (Figure 3) obtained from Nei’s unbiased genetic distance using the neighbor-joining method, in which medium and large Kari populations were closest to each other. All the three subtypes of Kari formed a distant cluster from Madakhlasht on the dendrogram.

**Ewens-Watterson test for neutrality**

Ewens-Watterson test was performed to detect selection in the populations, using the microsatellite data. Most of the markers were found neutral for selection in all the four sheep populations, having observed F-value within the 95% confidence intervals (Figure 4). The observed F-values of only two markers (INRA63 and BM1329) in small Kari, and three markers (OarFCB193, BM1824, and SRCRSP1) in large Kari population fall out of the limits of the confidence interval. In medium Kari and Madakhlasht populations, the observed-F values of all the markers fall within the upper and lower limits of 95% confidence intervals.

**Table 5.** Nei’s unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal) among populations

| Population       | Small Kari | Medium Kari | Large Kari | Madakhlasht |
|------------------|------------|-------------|------------|-------------|
| Small Kari       | ***        | 0.752       | 0.755      | 0.520       |
| Medium Kari      | 0.285      | ***         | 0.815      | 0.702       |
| Large Kari       | 0.280      | 0.205       | ***        | 0.656       |
| Madakhlasht      | 0.654      | 0.354       | 0.421      | ***         |

*Figure 2.* Summary plot showing the membership probability of individuals in the predefined populations at different K values. Each bar represents an individual. Note that at $K = 4$ a cluster made by medium Kari showed a proportion of small Kari in the ancestry; while an ancestral admixture of medium Kari and Madakhlasht was observed in the group made by large Kari.
Population bottleneck and mutation drift equilibrium
The heterozygosity excess/deficiency under different mutation models, as an indicator of mutation drift equilibrium, was calculated using the BOTTLENECK software (Table 6). The results showed that there were significantly higher numbers of loci with heterozygosity excess than expected under

Table 6. Values for mutation drift equilibrium in Kari subtypes and Madakhlasht sheep breeds under different mutation models and statistical tests

| Breed          | Mutation model | Hee  | He  | p   | T2  | p   | WS rank test |
|----------------|----------------|------|-----|-----|-----|-----|--------------|
| Small Kari     | IAM            | 12.46| 19  | 0.003 | 5.07 | 0.000 | 0.000 |
|                | SMM            | 12.74| 17  | 0.048 | 3.06 | 0.001 | 0.008 |
|                | TPM            | 12.58| 19  | 0.003 | 4.16 | 0.000 | 0.000 |
| Medium Kari    | IAM            | 13.47| 19  | 0.013 | 3.91 | 0.000 | 0.000 |
|                | SMM            | 13.59| 16  | 0.210*| 0.82 | 0.205*| 0.172* |
|                | TPM            | 13.48| 17  | 0.098*| 2.63 | 0.004 | 0.009 |
| Large Kari     | IAM            | 13.11| 20  | 0.002 | 4.19 | 0.000 | 0.000 |
|                | SMM            | 13.24| 18  | 0.032 | 1.70 | 0.044 | 0.007 |
|                | TPM            | 13.33| 18  | 0.035 | 2.99 | 0.001 | 0.000 |
| Madakhlasht    | IAM            | 8.64 | 17  | 0.000 | 5.75 | 0.000 | 0.000 |
|                | SMM            | 9.36 | 17  | 0.000 | 4.63 | 0.000 | 0.000 |
|                | TPM            | 9.23 | 17  | 0.000 | 5.05 | 0.000 | 0.000 |

Hee, expected number of loci with heterozygosity excess; He, number of loci with heterozygosity excess; p, probability value for heterozygosity excess; T2, test 2; WS, Wilcoxon-Sign; IAM, infinite allele model; SMM, stepwise mutation model; TPM, two-phase model.
all mutation models in all populations, except medium Kari. Similarly, test 2 (T2) values calculated for the populations under the standardized differences test was higher for all the populations, except medium Kari under the SMM model.

The results of the mode-shift test from the BOTTLENECK analysis, distributing the allele frequency data of the populations in different frequency classes, are graphically presented in Figure 5. The allele frequencies of the small Kari population were poorly distributed into different frequency classes, showing the presence of a large proportion of alleles of higher frequency class at several points. In contrast, the frequency distribution of medium and large Kari populations followed a normal L-shape graph showing a high proportion of alleles in low-frequency classes (0.01 to 0.20). A mode shift was also recorded in the Madakhlasht sheep population, showing a higher variation in the proportion of alleles in lower and upper-frequency classes and more distortion of normal L-shape compared to the small Kari population (Figure 5).

DISCUSSION

Population genetic diversity

Polymorphism information content: The markers used in the recent study are highly explicit ovine specific microsatellite markers recommended by the FAO [14]. Genetic markers are usually classified based on their PIC values, if the PIC value of a marker is less than 0.25, it is considered to be less informative while those whose PIC value is greater than 0.5 are considered to be highly informative for the quantitative genetic studies [17]. The PIC values between 0.25 and 0.5 are considered to be convincingly informative. In the current study, the mean PIC values calculated in the three subtypes of Kari sheep population (small, medium, and large Kari) were highly informative; however, in Madakhlasht sheep the PIC value was reasonably informative (0.462), suggesting a low level of genetic diversity and heterozygosity in this population.

The mean PIC values of the three subtypes of Kari sheep were in accordance with the Hazaragie sheep (0.534) of Afghanistan [23]. The PIC value of Madakhlasht sheep was in accordance with the other Pakistani sheep breeds Michni, Balkhi, and Hashtnagri [15]. One marker in large Kari (Oar FCB193) and one in Madakhlasht (SRCRSP9) could be considered less informative having PIC values less than 0.25. Another microsatellite marker (BM1824) has been found less informative previously in the Michni sheep population found in the central valley of KP, Pakistan [15].

Allele polymorphism: The Na observed in different subtypes of Kari sheep and Madakhlasht varied to a large extent. The lowest Na was observed in Madakhlasht and small Kari populations, suggesting the loss of respective alleles and genetic diversity in these populations probably due to the isolated habitat of these populations. The Na found in these populations (small Kari and Madakhlasht) was in accordance with the previously studied Pakistan sheep populations (Hashtnagri, Michni, and Balkhi) [15]. However, in two of the Kari subtypes (medium and large Kari), the Na was considerably higher, suggesting that Kari’s genetic diversity has been successfully maintained. The Na in medium and large Kari populations were in accordance with Afghan Hazaragie [23] and Indian Muzzafarnagri sheep [24]. Mean Ne and AR were less than Na in all the populations in this study, suggesting the predominance of certain alleles across the polymorphic loci in each of the populations [25].

Figure 5. Allele frequency distribution graph of the four sheep populations. A mode shift can be seen in small Kari and Madakhlasht populations, while medium and large Kari populations followed a normal L-shape graph.
Within the Kari subtypes, large and medium Kari shared a maximum number of alleles (57.6%) more than any other combination of the subtypes of Kari sheep in the current study. These two populations were also genetically close to each other as evident from the genetic distance between the populations. Madakhlasht population, which is genetically more distant from the Kari population, shared the least number of alleles (17.1%, 24.0%, and 29.7%) with small, medium, and large Kari, respectively. This may because of the geographical distribution of the Kari and Madakhlasht populations, where the three subtypes of Kari share the same breeding region (upper Chitral), while Madakhlasht inhabit a distinct location (lower Chitral). It should be noted that the altitude difference between lower and upper Chitral is about 6,000 m [4]. In our previous studies, three Pakistani sheep breeds Hashtnagri, Balkhi, and Michni populations found in adjacent breeding regions (in the central valley of KP) have been found to share more alleles (75% to 86%) [26].

The small Kari population can be distinctly characterized based on their higher number of unique alleles. Of the total unique alleles found in Small Kari in the current study, the frequency of five alleles exceeded 0.2. High-frequency unique alleles were also found in the other three populations in the current study. Such unique alleles can serve as genetic markers for the identity of a population, as previously suggested [27]. A higher number of unique alleles (12) have been found previously in the Pakistani Michni sheep population using the same microsatellite markers [26].

**Genotype heterozygosity and Hardy-Weinberg equilibrium**

When a population maintains its relative allele frequencies in succeeding generations, it is said to be in a state of HWE [28]. Deviation from HWE may be due to nonrandom matings, changes in allele frequencies between generations, the occurrence of mutation or migration, intensive selection or inbreeding, and changes in the heterozygosity [29]. The mean p-value for HWE in the current study revealed that all of the populations were in HWE; however, individual loci deviated from HWE in all the four populations, which may be due to a smaller number of alleles or heterozygosity at those loci. The presence of less heterozygosity than expected (Wahlund effect) is also one of the major causes of deviation from HWE [30]. This phenomenon was observed at several individual loci in all the three subtypes of Kari and one locus in Madakhlasht sheep. Mean heterozygosity was higher than expected in small Kari, large Kari, and Madakhlasht populations; however, it was less in medium Kari. These data indicate isolated subgroups in the medium Kari population as suggested in previous studies [25]. The average observed and expected heterozygosities in the sheep populations in this study were in accordance with Afghan Hazaragie sheep [23], and were higher than previously reported for Pakistani Michni, Balkhi, and Hashtnagri sheep populations [15].

To analyze whether the polymorphisms in the tested genomic regions (microsatellites) have been affected by the selection or not, the statistical test based on the algorithm proposed by Ewens and Watterson was used [31]. The results showed that most of the loci in all the four populations were neutral for selection, suggesting the absence of selection strategy or selective sweep in the populations [31]. Few of the microsatellite loci in small (two) and large (three) Kari populations, in the current study, could be identified as the regions affected by selection. These regions have been considered as a tool for the identification of specific differences between populations [32].

**Individual’s assignment to populations**

The principle of assigning an individual to a respective population is based on the probability of a genotype belonging to a sampled reference population [21]. Three main approaches are used: i) frequency-based method where the allele frequency of an individual is checked in the candidate population; ii) Bayesian method where the allele frequencies of the population are assessed for probable density at independent loci; iii) distance-based methods utilize the genetic distance between the individuals and the populations for the assignment. Different genetic distances have been used for this purpose (see Materials and Methods). In the current study, different methods showed varying percentage of correctly assigned individuals to their respective populations. The reliability of these methods depends on the conditions of the study.

For example, Cornuet et al [33] have reported that if a population is constantly changing in terms of mutations at a marker’s site then the Bayesian method is thought to be superior over the other methods. Contrary to this, if a population deviates from HWE and linkage disequilibrium, then the distance-based method is preferred over the Bayesian method. Similarly, if a population is genetically more diverse then the distances-based method is preferred [34].

Analyzing the situation of the present study, the genetic diversity of the populations was comparatively less than previously reported for other sheep breeds. Furthermore, most of these populations were found in HWE. These results indicate the suitability of the Bayesian and frequency-based methods, which showed a higher precision for the individuals belonging to small, medium, and large Kari sheep, indicating genetic differences between the three subtypes that had been grouped distinctly based on their phenotypes. Madakhlasht sheep, on the other hand, is more distinct (phenotypically and geographically) from the other three populations. Therefore, a distance-based approach for the assignment of Madakhlasht individuals can be considered more reliable, which showed
a correct assignment of a high percentage of Madakhlasht specimens. In our previous study, another Pakistani sheep breed (Michni) have been reported to have a higher assignment accuracy (82% to 98%); whereas, Hashtnagri sheep has been found to have less accuracy (2% to 88%) [26] compared to the current populations. The STRUCTURE analysis of the populations in this study further clarified the uniqueness of each of the four populations. The results showed a higher precision of individuals belonging to small Kari and Madakhlasht. The genotypes (individuals) of medium and large Kari populations were assigned jointly to two groups, indicating that these two populations share an admixed ancestry [22]. This further supported the low level of genetic differentiation and distance between medium and large Kari populations. The individuals belonging to small Kari made a separate cluster in the STRUCTURE analysis at all K values, suggesting strong genetic differentiation and a different ancestry of this population. Small Kari has previously been recognized to have a shorter gestation length compared to the medium and large Kari sheep [8]. Based on the structure analysis, Madakhlasht sheep also showed a distinct ancestry, which is in line with the greater phenotypic, geographical, and genetic distance of Madakhlasht from the three Kari populations.

**Interpopulation inbreeding and differentiation**

The pairwise $F_{ST}$ value equal to 0.05 suggests moderate differentiation among the populations [28]. Further lower values of $F_{ST}$ indicate a low level of differentiation. In the current study, $F_{ST}$ values among the pairs of the four sheep populations were found more than 0.05, which indicated a high level of differentiation between the populations. The values of $F_{ST}$ between pairs of the three Kari populations were less than between any of the Kari subtypes and Madakhlasht. This and other analyses discussed above suggest that Madakhlasht is a different breed and is not related to Kari. The higher $F_{ST}$ values between the small Kari and the other Kari subtypes were in accordance with its genetic distinctness identified in the STRUCTURE analysis in this study.

In our previous studies, other well-differentiated Pakistani sheep breeds have shown lower $F_{ST}$ values (0.081 between Hashtnagri and Michni, and 0.076 between Balkhi and Michni [26]), which further indicate separate identities of the populations in this study. Similar $F_{ST}$ value (0.118) have been reported between Indian Muzzafarnagri and Marwari sheep populations [24]. Population differentiation ($F_{ST}$) may diminish due to extensive crossbreeding and sharing the same breeding region, as has been observed in our previous study between Balkhi and Hashtnagri ($F_{ST} = 0.043$) sheep populations [26].

Total inbreeding ($F_{IT}$) and the gene flow ($N_{m}$) between the populations in this study were lower compared to the previous studies on other Pakistani sheep breeds, Balkhi, Hashtnagri, and Michni [26]. The $F_{IT}$ and $N_{m}$ were higher between medium and large Kari populations compared to other combinations. Having a similar phenotype and geographical distribution, the possibility of inbreeding between these two populations could not be excluded. Medium and large Kari populations also showed less genetic distance and more ancestral admixture in the current study, which may because of the higher level of inbreeding and gene flow between the two populations. The lower values obtained for $F_{IT}$ and $N_{m}$ between Madakhlasht and the Kari subtypes further affirmed the genetic remoteness of Madakhlasht sheep.

**Population bottleneck and mutation drift**

As a population’s size reduces, its number of alleles reduces faster than the heterozygosity at the given loci, resulting in higher heterozygosity than expected and causing a genetic bottleneck [20]. If a population can maintain its allele number, it is said to be in mutation drift equilibrium. Bottleneck software provides the opportunity of computing Mutation drift equilibrium using the allele frequency data under different mutation models. In SMM the changes in fragment length (increase or decrease in allele size) is exploited; TPM considers substitution mutation having no change in allele size; while the IAM (Crow and Kimura model) considers heterozygosity as a function of consistent changes in allele frequency in a population under the consistent pressure of mutation and subsequent elimination dominated by mutation drift. For microsatellite data, the results obtained under SMM are more acceptable compared to the other two mutation models [35].

The software evaluates these mutation models using different statistical tests (see Materials and Methods). These tests showed a significantly higher number of loci with heterozygosity excess than expected in all the populations in this study except for medium Kari under SMM, where the observed and expected values for heterozygosity excess were statistically similar. These results suggest the absence of a genetic bottleneck in the medium Kari population, indicating that this population has been able to maintain its population size in the recent past. This was further confirmed by the lower T2 value (less than 1.6 [36]) for medium Kari, and by the mode shift test which followed a normal L-shape graph.

The large Kari population also showed a lack of genetic bottleneck indicated from the number of loci with heterozygosity excess as expected ($p = 0.032$ and 0.044), lower T2 value (1.7), and a normal L-shape graph. In the small Kari population, the observed number of loci with heterozygosity excess can be considered similar to the expected value ($p = 0.048$ under sign test); however, higher T2 value and distortion of allele frequency graph may indicate the pres-
ene of genetic bottleneck in this population. Madakhlasht population, on the other hand, can be said to have lost its population size because of having a higher number of loci with heterozygosity excess than expected, distorted allele frequency distribution graphs, and higher T2 values.

Whatever the ground realities maybe, two reasons can be speculated for the presence of genetic bottleneck in the small Kari and Madakhlasht populations. i) Crossbreeding: as the animals belonging to the two populations are small in size; the farmers may have adopted crossbreeding them with other larger breeds. ii) Intensive inbreeding: these populations may be intensively inbred because of inhabiting an isolated breeding region (as in the case of the Madakhlasht population). The unique genetic makeup of these breeds requires their conservation on a priority basis to preserve their valuable characteristics (e.g. shorter gestation length in small Kari) for future breed improvement programs.

CONCLUSION

Kari is one of the thin tail sheep breeds of the Khyber Pakhtunkhwa that can be divided into three subtypes based on their phenotype and genetic makeup. Among these subtypes, each possesses unique alleles that could serve as genetic markers. Medium and large Kari populations have been able to maintain their population size; however, small Kari seems to have lost its population size showing the presence of a bottleneck. Madakhlasht can be considered as a different breed having unique genetic makeup; however, it was also found to be in a genetic bottleneck. The data suggest further study of these breeds for variation in their specific characteristics such as productivity and reproduction, and exploitation of their unique values for improving local sheep populations.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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