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Evaluation of Genetic Effects of Demographic Bottleneck in Muzzafarnagri Sheep from India Using Microsatellite Markers

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ABSTRACT : Genetic variability is an important component in the ability of populations to adapt in the face of environmental change. Severe human impacts reduced Muzzafarnagri sheep of India from 500,000 in 1972 to 10,989 in 1973-74. Here we report for the first time the effect of this population decline on levels of genetic variability at 13 FAO recommended ovine microsatellite loci and contrast levels of variability to that in a breed from the same geographical region, which differed in numbers, by an order of magnitude (Marwari sheep). Of the 13 loci, 100% were polymorphic in both breeds. A high degree of genetic variation was observed within populations in terms of both allele diversity (number of alleles per locus, >4) and gene diversity (expected heterozygosity, >0.5), which implied that there is still a substantial amount of genetic diversity at the nuclear loci in a declining population. Nevertheless, overall low number of alleles per locus and relatively less abundance of low frequency alleles in Muzzafarnagri sheep suggested that genetic variability has been comparatively reduced in this population. Bottleneck analysis indicated that a genetic bottleneck did not occur during the most recent decline. In addition, we found that the differentiation among populations was moderate (F_{ST} = 11.8%). This study on assessment of genetic effects of the population declines in ovines is a step towards identification of genetically impoverished or healthy populations, which could prove to be a useful tool to facilitate conservation planning in this important species of small ruminants. (**Key Words :** Microsatellite, Genetic Variability, Population Decline, Muzzafarnagri Sheep)

INTRODUCTION

Human activities in the past few hundred years have caused enormous impacts on many ecosystems, greatly accelerating the rate of population decline and extinction. Severe population declines or bottlenecks can have a profound impact on effective population size and the maintenance of genetic variation within a population. Because bottlenecks may influence the distribution of genetic variation within and among populations, the genetic effects of reductions in population size have been studied extensively by evolutionary biologists (Nei et al., 1975). More recently, conservation biologists have become concerned with the effects of demographic bottlenecks on the viability of small populations. Loss of genetic diversity may reduce the potential of small populations to respond to selective pressures (Allendorf and Leary, 1986), and increased inbreeding may reduce population viability (Westemeier et al., 1998). A common theme in conservation genetics has been the use of genetic variation to identify populations that have experienced demographic bottlenecks. Populations known to have experienced a reduction in demographic size often show reduced genetic diversity. Numerous threatened or endangered species and populations have been found to have low levels of genetic variation (O'Brien, 1994). Using the converse of the theory that bottlenecks result in the loss of genetic diversity, low genetic diversity has often been taken as evidence that a population has experienced a bottleneck. However, not all populations that have been reduced to small sizes show measurably lower levels of nuclear genetic diversity (Bowling and Ryder, 1987).

In order to make biologically sound conservation plans, an understanding of the genetic diversity remaining in natural populations is essential (Tallmon et al., 2002). Unfortunately, it is often difficult to identify losses of variability because levels of genetic variability prior to a population decline are generally unknown (Spencer et al., 2000). A number of statistical methods now make it possible to investigate a population's history without the need for information on past population sizes (Spencer et al., 2000). These tests typically quantify deviations from expected patterns in allele sizes, allele numbers,

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Table 1. Genetic variability measures and an evaluation of informativeness of microsatellite loci (PIC) in Muzzafarnagri and Marwari sheep

Locus	Chr. location	Observed allele No.		Expected heterozygosity		Polymorphism information content (PIC)		HWE (P)		Allele size range (bp)
		Mu	Ma	Mu	Ma	Mu	Ma	Mu	Ma	
BM757	9	4	6	0.596	0.786	0.534	0.783	0.443	0.078	176-202
BM827	3	4	6	0.636	0.821	0.568	0.647	0.193	0.139	212-222
BM1314	22	7	9	0.811	0.824	0.774	0.804	0.116	0.001*	149-183
BM8125	17	5	6	0.713	0.756	0.667	0.721	0.356	0.432	108-120
CSSM47	2	3	4	0.595	0.305	0.514	0.288	0.003*	1.000	138-182
OarAE129	5	4	4	0.699	0.563	0.646	0.521	0.000*	0.000*	146-164
OarCP34	3	7	5	0.807	0.720	0.779	0.674	0.006*	0.124	114-128
OarFCB128	2	6	4	0.822	0.694	0.796	0.639	0.118	0.055	100-132
OarHH35	4	6	9	0.774	0.835	0.739	0.816	0.423	0.000*	128-160
OarHH64	4	6	8	0.772	0.795	0.739	0.775	0.000*	0.000*	118-138
OarJMP8	6	7	6	0.817	0.788	0.739	0.839	0.613	0.016*	117-135
OarVH72	25	5	5	0.671	0.530	0.628	0.491	0.786	0.004*	127-141
RM4	15	6	8	0.779	0.802	0.746	0.779	0.468	0.004*	139-159
Mean		5.38	6.15	0.730	0.709	0.682	0.675			

Mu = Muzzafarnagri, Ma = Marwari, * $p<0.05$.

heterozygosity levels, or allele distributions, often using microsatellites data as these molecular markers are important modern tools for estimating the level of genetic diversity in imperiled populations (O'Brien, 1994). Clearly an evaluation of approaches to assess bottlenecks with molecular markers would aid conservation and evolutionary geneticists in studying reductions in population size (Spencer et al., 2000). These methods hold promise because information about a population's past history is often useful when predicting future population dynamics and extinction risks resulting from demographic or genetic processes.

Muzzafarnagri sheep, the largest and heaviest mutton breed is well adapted to the hot and humid irrigated region of Northwestern semi-arid zone of India. The fast body growth coupled with high feed efficiency is its main characteristics. This breed is under constant risk due to small grazing area, reflection of increasing human population pressure and more and more land coming under crop production. There was a severe decline in the population of the breed in its home tract from 1972-1973 onwards. The population of this breed was 500,000 in 1972 and 10,989 in 1973 according to survey carried out by the Uttar Pradesh Government. The population in 1973-74 was only 0.11 percent of the total sheep population (Mandal et al., 2000). Moreover, the population has been reported to be 6,475 in the year 2003 by sheep and wool extension centers of U.P Government, in Muzzafarnagar District-the central habitat of the breed. The latest sheep population figures of this breed are, however, not available, but it is apparent that numbers are declining due to natural reasons and human impacts. Habitat fragmentation and reduced population size all raise the concern that populations will become genetically impoverished, and thus unable to adapt to future environmental changes and to develop resistance to new diseases.

In this study efforts were made to compare the genetic diversity of declining population of Muzzafarnagri to a large relatively undisturbed population of Marwari sheep (1972 census: 4.367 m; 1977: 5.018 m) of the same North western arid and semi arid region of India. The goal is to have a better under standing of human impacts on genetic diversity in Muzzafarnagri sheep since genetic diversity is a primary component of adaptive evolution.

MATERIAL AND METHODS

Sample collection and DNA extraction

A total of 82 blood samples were randomly collected from genetically unrelated animals across their breeding tract. Samples for the 34 individual Muzzafarnagri sheep used in this study were collected from Muzzafarnagar district in Uttar Pradesh state and also from the flock maintained in the farm at Indian Veterinary Research Institute, Bareilly. Blood samples of 48 Marwari sheep from villages in and around Nagour district in Rajasthan state – its home tract, were used for comparison with declining Muzzafarnagri population. Blood sampling was coordinated with owners and veterinary officers. DNA extractions followed a standard phenol/chloroform protocol.

Microsatellite markers and genotyping

Thirteen unlinked polymorphic microsatellite loci (Table 1) recommended in MoDAD project of FAO (1996) for sheep genetic diversity studies were scored. Typical polymerase chain reaction (PCR) testing was carried out under these conditions: 60 ng of target DNA was used in 25-μl PCR reaction containing 1×PCR buffer, 50 ng of each primer, 200 μM of dNTPs, 0.5 units of Taq DNA

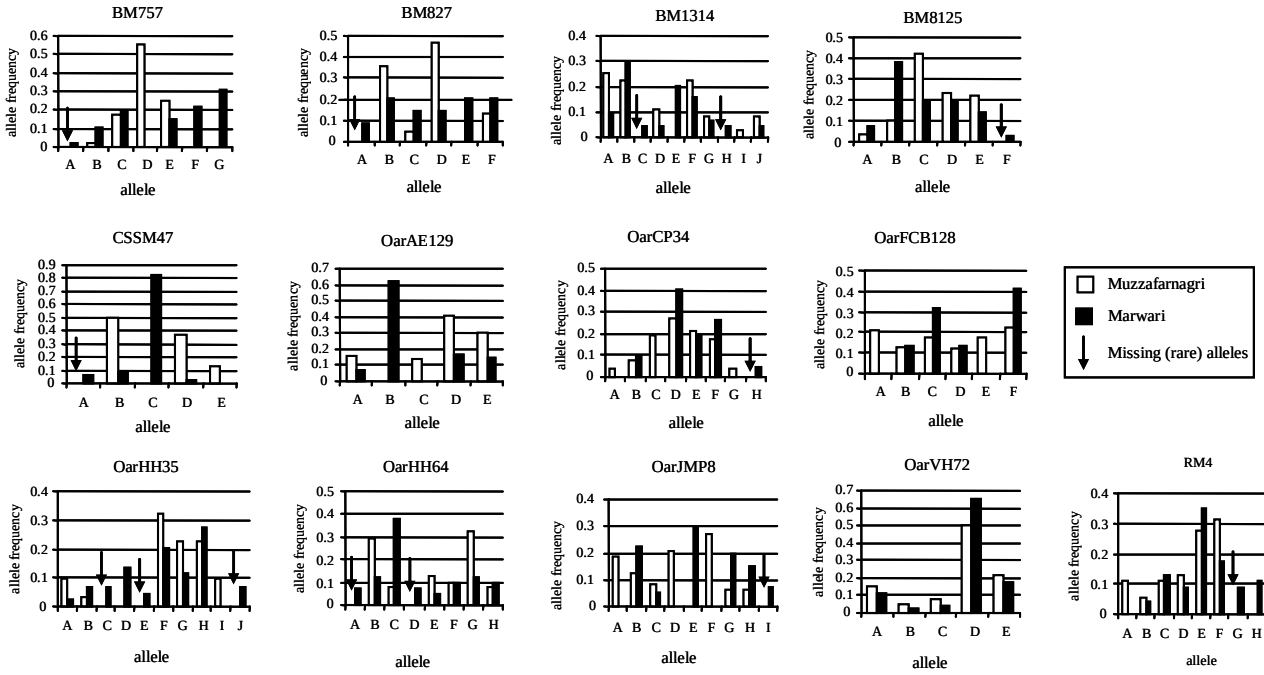

Figure 1. Frequency histogram for 13 polymorphic microsatellite loci in Muzzafarnagri and Marwari sheep.

Polymerase and 1.5 mM $MgCl_2$. A Common "Touchdown" PCR profile included 3 cycles of 45 sec at 95°C, 1 min at 60°C; 3 cycles of 45 sec at 95°C, 1 min at 57°C; 3 cycles of 45 sec at 95°C, 1 min at 54°C; 3 cycles of 45 sec at 95°C, 1 min at 51°C and 20 cycles of 45 sec at 92°C, 1 min at 48°C (FAO, 1996). Alleles were scored using unlabeled primers with products visualized by silver staining (Bassam et al., 1991). Allelic size range (Table 1) was estimated using 10 bp DNA ladder (Gibco BRL, life technologies, TM). Genotype of individual animal of the two breeds at 13 microsatellite loci was recorded by direct counting.

Data analysis

Genetic variation analysis/levels of variation : Genotypic data were analyzed using POPGENE (Yeh et al., 1999) to calculate the allele frequencies, observed number of alleles, effective number of alleles, observed heterozygosity and expected heterozygosity (Nei's, 1973). Polymorphism information content values (PIC) were estimated according to Botstein et al. (1980).

We tested changes in mean heterozygosity and mean number of alleles per locus (allelic diversity) using a Wilcoxon signed rank test, which pairs the data by locus. We also examined allelic richness, which is another measure of allelic variation that takes into account unequal sample sizes using the technique of rarefaction (Petit et al., 1998). Allelic richness was calculated with the program FSTAT (Goudet, 1995). Deviations from Hardy-Weinberg equilibrium were assessed by GENEPOP (Raymond and Rousset, 1995).

Population differentiation : Population differentiation was assessed using the microsatellite-based measure of differentiation-F_{ST} calculated with the program FSTAT (Goudet, 1995).

Bottleneck detection : A number of tests based on microsatellite data have been developed for the detection of bottleneck events in a population's past. Allele frequency distribution of the microsatellite loci was examined by using program Bottleneck 1.2.02, for mode shift (Luikart et al., 1998a), which may indicate if a recent genetic bottleneck has occurred.

RESULTS AND DISCUSSION

This paper represents the first attempt to assess the genetic effects of the population decline in Muzzafarnagri breed of sheep in India. Using a model based on levels of variability in Marwari we assessed the extent to which levels of genetic variability have been lost in Muzzafarnagri. Microsatellite markers were selected because they are useful for detecting populations that have undergone severe declines (Spencer et al., 2000).

Pattern of variation across breeds

Muzzafarnagri population was assessed for a deficiency of low frequency allele classes relative to Marwari, as populations that have undergone severe size reductions for protracted periods are expected to show a reduced abundance of low frequency allele classes (Luikart et al., 1998b). This reduction or 'mode shift' represents the loss of

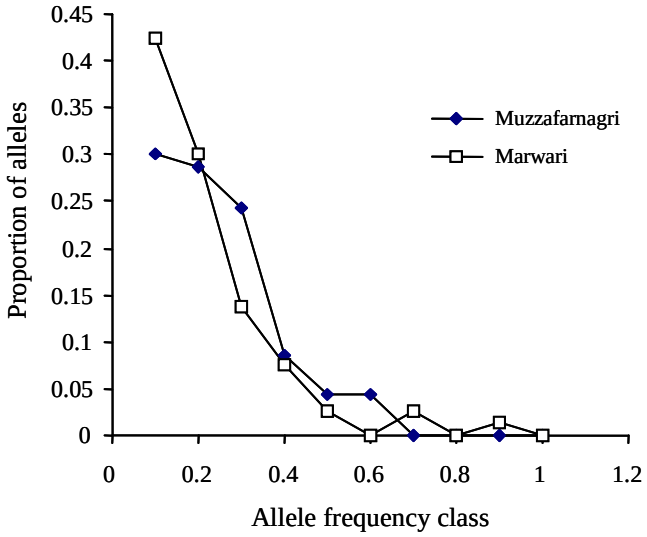

Figure 2. Mode-shift test for Bottleneck in Muzzafarnagri and Marwari sheep.

rare alleles and may be assessed by looking at the overall distribution of allele frequency classes. The number of alleles present for all the 13 polymorphic loci (polymorphism depicted by Polymorphism information content values, PIC; Table 1) and their frequencies in the complete sample of sheep (Muzzafarnagri, N = 34, Marwari, N = 48) represented graphically in Figure 1, showed a relative deficiency/uncommonness of rare alleles (i.e., frequency less than 0.1) in Muzzafarnagri sheep. Among all 13 loci, 15 rare alleles detected in Marwari population were not discerned in the declining Muzzafarnagri population (Figure 1). These results are consistent with those of several other studies of endangered/declining populations of various animal species (Waldick et al., 2002; Bellinger et al., 2003). These findings suggested that genetic variability has been reduced in this population as both the used /investigated sheep breeds were from the same Northwestern semi-arid zone of India which minimized the differences in allele frequencies arising from geographic rather than temporal differences (Waldick et al., 2002).

In both the breeds, deviation from the Hardy-Weinberg equilibrium was discerned at several microsatellite loci. The Marwari showed significant ($p<0.05$) deviation from Hardy–Weinberg equilibrium at seven loci, followed by the Muzzafarnagri (four loci). Similar results have been reported from a wide range of other animal species (Bentzen et al., 1996; Bagley et al., 1999). This deviation might represent either of the listed biological phenomenon viz., over dominant selection, inbreeding, small population size, non-random mating of individuals and linkage disequilibrium. However, there did not appear to be significant deviations from equilibrium across all loci within any population, subsequent analyses were carried out on the basis that Hardy-Weinberg equilibrium prevailed.

For further comparative purposes, the number of observed alleles (allele diversity), expected heterozygosity (gene diversity) and polymorphism information content values for Muzzafarnagri and Marwari breeds, estimated for same set of 13 microsatellite loci, are shown in Table 1. In general, allele sizes in the sample were similar to those described for the same loci in other ovine breeds (Sodhi et al., 2003; Arora and Bhatia, 2006). Apart from one locus (CSSM47), which exhibited 3 alleles in the Muzzafarnagri breed, we observed a high degree of genetic variation within Muzzafarnagri investigated populations in terms of allele diversity (>4 alleles per locus). A total of 94 different alleles were observed at all seven loci in the two populations. Nevertheless, overall low number of alleles per locus in Muzzafarnagri sheep (5.38) in comparison to Marwari sheep (6.15) supported reduction in genetic variability of this declining breed. This loss of genetic variation following a population decline is in agreement with similar observations in other animal species (Bellinger et al., 2003).

The observed (Ho, not shown in Table 1) and expected (He) heterozygosity values were high (>0.5) for all loci. The low He value for locus CSSM47 (He = 0.305) in Marwari population may be attributed to the presence of null alleles. We used the latter (He) in our analyses, as it is considered to be less biased and a better estimator of the genetic variability present in a population (Kim et al., 2002). Estimates of He for all the 13 polymorphic loci ranged from 0.595 (CSSM47) to 0.822 (OarFCB128), with a mean of 0.730 in declining Muzzafarnagri and 0.305 (CSSM47) to 0.835 (OarHH35), with a mean of 0.709 in undisturbed population of Marwari sheep. Similarly allelic richness was also high in both the breeds (Muzzafarnagri = 5.28, Marwari = 6.00). Nevertheless, no significant variations were discerned between the breeds at allele ($p = 0.1294$) and gene ($p = 0.7148$) diversity levels using Wilcoxon sign rank test, as this test in a recent comparative analysis of statistical methods has been observed to perform better in identifying bottlenecked populations (Maudet et al., 2002). The results from this study indicate that there is still a substantial amount of genetic diversity at the nuclear loci in declining Muzzafarnagri population.

Degree of genetic divergence between populations

The F_{ST} value (0.118) indicated a non significant genetic divergence between the two populations ($p>0.05$), Present values are lower than those of Korean and Chinese domestic goats (F_{ST} = 20.2%, Kim et al., 2002), Asian goat populations (F_{ST} = 14.3%, Barker et al., 2001) higher than that of Trans Caucasian native sheep breeds (F_{ST} = 9%, Hirbo et al., 2006), Indian indigenous sheep breeds (F_{ST} = 8.3%, Sodhi et al., 2006) and almost similar to other domestic animals viz; Indian cattle breeds (Fst = 11.3%, Mukesh et al., 2004). Obtained results suggested moderate

genetic differentiation between investigated sheep populations (Hartl, 1980).

Genetic bottleneck analysis

The bottleneck test for a mode shift in allele frequency classes with 13 microsatellite loci as per earlier recommendations of 8-10 loci (Spencer et al., 2000) found both populations to have L-shaped distributions consistent with normal frequency class distribution ranges ($p>0.05$, Figure 2, Waldick et al., 2002). This finding suggested the absence of any detectably large, recent genetic bottleneck (last 40-80 generations) in declining Muzzafarnagri and relatively undisturbed Marwari sheep populations.

Our study demonstrates that although genetic variability was not reduced significantly in Muzzafarnagri sheep, there are indications that some variability has been lost in this important indigenous Indian breed of sheep. Investigations are needed to identify genetically healthy or impoverished ovine populations that appear to be facing demographic declines (Kuo and Janzen, 2004) since this decline in genetic diversity would inevitably lead to a decrease in fitness (Beheregaray et al., 2003). Furthermore, susceptibility to environmental stochasticity viz., any increase in extrinsic stresses such as food shortages, habitat loss/disturbance, or increased mortality associated with human activities etc. would result in an increase in their extinction probability. Microsatellite based genetic diversity data could be a useful tool to help conservation planning in this regard as fewer efforts will be required to conserve a genetically healthy population rather than a genetically impoverished one.

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