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Molecular genetic architecture analysis of Nellore sheep (*Ovis aries*) revealed through short tandem repeat markers

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Abstract
The objective of the present investigation is to employ the Nellore Sheep population's genetic analysis at the molecular level to create sustainable advancements, conservation, and utilization of the breed, all of which are capable of improving the quality of life for all involved. The Food and Agricultural Organization, Rome, Italy, recommended the use of 25 short tandem repeat markers for the genetic characterization and bottleneck analysis of Nellore Sheep. This was achieved by isolating genomic DNA from blood samples of 65 unrelated Nellore Sheep with typical phenotypic features collected from various villages in the breeding tract. Bioinformatics software has been employed to calculate the fundamental measures of genetic variation. Three tests were conducted under three different mutation models—the infinite allele model (IAM), the stepwise mutation model (SMM), and the two-phase model (TPM)—in order to assess the Nellore Sheep for mutation drift equilibrium. Additionally, the expected equilibrium gene diversity (H_{eq}) and observed gene diversity (He) were estimated under various microsatellite evolution models. The analysis showed that there were 162 alleles overall, with a mean of 6.48±1.75 alleles across loci, and that the observed number of alleles varied from 3 (SRCRPS5) to 11(OarJMP29). High genetic diversity was indicated by the total observed heterozygosity, expected heterozygosity, inbreeding estimate, and polymorphism information content values which were 0.6580±0.2065, 0.7591±0.099, 0.233±0.044, and 0.7105±0.023 respectively. In accordance to the IAM, TPM, and SMM models, there were 18, 07, and 12 locations that have been shown to exhibit an excess of gene diversity, respectively. Under the IAM and TPM models, there was a substantial deviation of sheep from the mutation-drift equilibrium as revealed by all three statistical tests (sign test, standardized differences test, and Wilcoxon sign rank test); under the SMM model, there was a non-significant deviation. The results under SMM showing the absence of the genetic bottleneck in sheep in the recent past have been verified by the qualitative test of mode shift analysis.

Keywords: Bottleneck, genetic diversity, microsatellites, Nellore sheep

Introduction
India has been blessed with an abundance of native sheep, including 45 recognized varieties that have been developed over several generations by farmers for an abundance of purposes. The overwhelming majority of these breeds were developed for wool and meat. All of these breeds have developed tolerance for tropical heat, are comparatively more resistant to tropical diseases, and do well on limited feed, primarily crop leftovers. They are also well suited to the agro climatic conditions that prevail in their respective native tracts. Nonetheless, over the past several decades, a few of these native sheep have dwindled as a result of shifting agricultural practices (Rout *et al.* 2008, Arora *et al.* 2011[a], [b],Mahmoudi *et al.* 2012, Mukhongo *et al.* 2014) [26, 4, 5, 17]. Microsatellites have shown to be an effective tool in detecting genetic links across various cattle breeds worldwide in recent years. Microsatellites show higher levels of variation, which makes more straightforward to recognize population differentiation. Because microsatellites exhibit larger degrees of variation, it is possible to identify population differentiation more effectively, which aids breeders in making informed decisions for the preservation and enhancement of priceless Germplasm (Arora and Bhatia, 2006, Arora *et al.* 2011a,b) [3, 4, 5]. Based on microsatellite markers, various studies have been conducted on the genetic diversity of sheep. The Nellore sheep is a huge individual with a diminutive body.

They have a uniformly creamy white color and a black ventral area that stretches from the inner side of the jaw to the inguinal region which is native to the state of Andhra Pradesh. This meat breed plays an important part in southwest part of Tamil Nadu. Small-scale and marginal farmers as well as laborers without access to land largely raise this breed for the purpose of producing meat. Due to the extremely disorganized nature of their breeding, immediate effort is required to both maintain and enhance these sheep's genetic makeup. The bottleneck analysis and genetic architecture of the sheep population have not been studied in their native environment. In order to assess genetic variation at the molecular level and look for evidence for recent population bottlenecks in sheep, microsatellite analysis was undertaken.

Materials and Methods

Blood samples from 65 unrelated sheep with typical phenotypic traits from several villages in the breeding tract were used to isolate the genomic DNA (Miller *et al*. 1988). After extensive conversations with the farmers, animals were selected from various villages to ensure unrelatedness even in the absence of parentage documents. Allelic data were generated using a battery of 25 sheep-specific microsatellite markers (BM757, BM827, BM1329, BM6506, BM6526, BM8125, CSSM31, ILSTO33, MAF65, MAF70, MAF209, MCM527, OarCP20, OarFCB20, OarCP34, OarFCB48, OarFCB128, OarFCB304, OarAE129, OarJMP29, OarHH35, OarHH47, OarVH72, SRCRSP5 and SRCRSP9). In a thermal cycler (BioRad, Germany), the microsatellite loci were amplified using the polymerase chain reaction (PCR) under the conditions: 5 minutes at 95 °C, 35 cycles of 50 seconds at 96 °C, 50 seconds at an annealing temperature (based on the locus), 45 seconds at 74 °C, and a final extension at 74 °C for 15 minutes. Using an automatic sequencer, capillary electrophoresis was used to genotype microsatellite markers, and Applied Biosystems, India's ABI 3130 was used to record the results. Version 4.0 of the data gathering software. The Microsatellite Analyzer 3.15 version was utilized to calculate the fundamental metrics of genetic variation (Peakall and Smouse, 2006) [20]. Using Arlequin version 3.1, the Hardy–Weinberg equilibrium test was carried out precisely (Excoffier *et al.* 1992) [9]. The GenAlEx tool was used to estimate the content of polymorphism information (Raymond and Rousset 1995) [25]. The sheep population's mutation drift equilibrium was evaluated using statistical methods, utilizing the fluctuation between allelic diversity and heterozygosity as the foundation. To determine the significant number of loci with heterozygosity excess, three tests namely the sign test, the standardized differences test, and the Wilcoxon sign rank tests were conducted under three different mutation models. Using the DISPAN program (Ota, 1993) [19], phylogenetic trees were constructed and the DA genetic distance (Nei *et al*., 1983) [18] was computed. Using the Bayesian clustering approach of STRUCTURE ver 2.3 (Pritchard *et al*. 2000) [22], the genetic makeup and degree of mixing of four sheep populations were examined.

We conducted 50 separate runs for every K value between 2 and 11. We utilized STRUCTURE Harvester (Earl and von Holdt, 2012) [8], which implements the Evanno approach (Evanno *et al*., 2005) [10], to determine the most likely groups (K) that best suit the data. To align the 50 repetitions of each K, the program CLUMPP ver 1.1 (Jakobsson and Risenberg, 2007) [12] was utilized. Next, the cluster visualization program DISTRUCT ver 1.1 (Rosenberg, 2004) [23] was run with the output from CLUMPP as an input. In addition, a qualitative test of mode shift has been carried out according to the BOTTLENECK tool to assess the stochastic distribution of alleles at various microsatellite loci.

Results and Discussion

Each of the 25 microsatellite loci successfully amplified in sheep breeds, generating distinct patterns that allowed genotype identification at the individual level. Four loci alone revealed substantial deviations in the checking for disequilibrium relationships between the various combinations of loci under examination; these loci were eliminated, leaving the other 25 loci intact for research. Table 1 displays the fundamental measures of genetic diversity. According to the study, 162 different alleles spread over 25 loci were found. With a mean of 6.48 ±1.75, the allelic polymorphism varied from 3 (SRCRSP5) to 11 (oarJMP29) (Kumarasamy *et al*. 2009, Pramod *et al.* 2010, Mukhongo *et al.* 2014) [13, 17]. The average observed number of alleles found in this research was comparable to the worth of 10.20 found in Madras Red Sheep and greater than the values found in Kilakarsal and Coimbatore sheep. The lack of selection pressure and random sample selection are likely to blame for the great allelic diversity seen (Takezaki and Nei., 1996) [28]. The heterozygosities that were observed and expected were 0.6580±0.2065 and 0.7591±0.099 on average (Arora and Bhatia 2004, Sharma *et al.* 2010, Yadav *et al.* 2011, Mukhongo *et al.* 2014) [2, 27, 29 17]. The polymorphism information content (PIC) values of gene diversities for the genetic markers are calculated under the three mutation models, SMM, TPM, and IAM. The expected gene diversities (Heq), estimated under three models of microsatellite evolution, were compared to the observed gene diversity (He) to ascertain whether there is an excess or deficiency of gene diversity at each location within the sheep population. Three statistical tests were performed for each mutation model, and the results showed that the Sign rank test rejected the null hypothesis of mutation drift equilibrium and showed a significant heterozygosity excess under IAM and TPM (Bhatia *et al*. 2008, Pramod *et al*. 2009, Mukhongo *et al*. 2014) [6, 21, 17]. However, in case of SMM, though heterozygosity excess was found in 12 of the 25 loci studied, it was not significant (Table-2). Under the IAM and TPM models, the standardized difference test results revealed positive and significant T2 values; however, under the SMM model, the results were negative and non-significant. Similar results were obtained using the Wilcoxon sign rank tests, with a significant heterozygosity excess under the IAM and TPM models and a non-significant result under the SMM model (Molaei *et al*. 2011, Dashab *et al.* 2011) [16, 7]. The three tests in the current analysis showed a significant departure under the sign and standardized differences test and a non-significant divergence under the SMM model. IAM predicts less equilibrium gene diversity than SMM, hence for any given data set, it is more likely to have a significant heterozygosity excess. Sheep were found to diverge from equilibrium gene diversity under the IAM and TPM models, but not under the statistically more conservative SMM model, which suggested that there was no genetic basis for the

demographic bottleneck. One other method for identifying potential bottlenecks was the mode-shift indicator test. This method uses qualitative analysis to pinpoint the genetic bottleneck. In populations that are not bottlenecked, a significant proportion of unusual alleles are expected (Bhatia *et al*. 2008, Pramod *et al*. 2009, Mukhongo *et al*. 2014) [6, 21, 17]. Because of bottleneck events, it is predicted that rare or low frequency alleles will become less common in the population than alleles with intermediate frequencies. Under these conditions, a mode shift away from the usual L-shaped distribution will occur when plotting the proportion of different alleles against allele frequency classes. The frequency distribution of alleles in the sheep population showed a typical L-shaped curve, with the most common alleles being those with the lowest frequency (0.001–0.1). This distribution unequivocally demonstrates that there hasn't been a recent bottleneck in the population under study. The possibility of a genetic bottleneck based on microsatellite data has generally not received much attention in research examining African and African cattle breeds such as Dexter and Bargur, as well as Valais Black neck sheep (Evanno *et al.,* 2005) [10]. Previous research on various Iranian and Indian sheep breeds could not find any evidence of the genetic bottleneck's recent appearance.

Table 1: Effective number of alleles, observed and expected heterozygosities, polymorphism information content (PIC), Shannon's information index, $F_{IS,}$ Hardy-Weinberg equilibrium (HWE) $\chi 2$ values and Nei's genetic distance values for Nellore sheep

Microsatellite Marker	n_a	n_e	H_o	H_e	PIC	I	F_{IS}	HWE		D
								χ^2	d.f	
BM757	4	3.1858	0.8000	0.6977	0.6317	1.2588	-0.1660	4.73^{NS}	6	0.6861
BM827	6	5.4878	1.0000	0.8316	0.7923	1.7465	-0.2228	4.73*	15	0.8178
BM1329	5	4.3584	0.8333	0.7836	0.7346	1.5388	-0.0815	4.73*	10	0.7706
BM6506	6	4.2155	0.9000	0.7757	0.7283	1.5961	-0.1799	30.64*	15	0.7628
BM6526	7	3.5019	0.8000	0.7266	0.6691	1.4661	-0.1198	9.61^{NS}	21	0.7144
BM8125	7	3.1328	0.6400	0.6947	0.6298	1.4119	0.0599	201.48*	21	0.6808
CSSM31	8	5.3412	0.6000	0.8266	0.7886	1.8367	0.2618	201.48*	28	0.8128
ILSTO33	8	4.3478	0.5333	0.7831	0.7440	1.7147	0.3074	201.48*	28	0.7700
MAF65	8	4.3478	0.8333	0.7831	0.7480	1.7682	-0.0823	201.48^{NS}	28	0.7700
MAF70	4	2.5496	0.6071	0.6188	0.5371	1.0555	0.0010	201.48*	6	0.6078
MAF209	7	5.8074	0.5714	0.8429	0.8058	1.8441	0.3097	201.48*	21	0.8278
MCM527	6	3.8793	0.5000	0.7548	0.6995	1.4998	0.3263	33.85*	15	0.7422
OarCP20	7	6.1612	0.8621	0.8524	0.8163	1.8622	-0.0291	33.85*	21	0.8377
OarFCB20	8	4.6392	0.9333	0.7977	0.7557	1.7327	-0.1898	33.85^{NS}	28	0.7844
OarCP34	5	4.2609	0.7143	0.7840	0.7298	1.5308	0.0667	33.85*	10	0.7653
OarFCB48	9	5.0992	0.7667	0.8175	0.7841	1.8996	0.0463	33.85*	36	0.8039
OarFCB128	7	5.6306	0.4400	0.8392	0.8006	1.8405	0.4650	33.85*	21	0.8224
OarFCB304	6	4.1634	0.4828	0.7731	0.7262	1.5848	0.3646	33.85*	15	0.7598
OarAE129	4	2.2167	0.3667	0.5582	0.5074	1.0371	0.3320	33.85*	6	0.5489
OarJMP29	11	8.6957	0.7333	0.9000	0.8467	2.2549	0.1714	33.85*	55	0.8850
OarHH35	7	3.6000	0.4333	0.7345	0.6818	1.5140	0.4000	33.85*	21	0.7222
OarHH47	6	4.0359	0.7667	0.7650	0.7169	1.5668	-0.0192	33.85*	15	0.7522
OarVH72	6	3.9474	0.3000	0.7593	0.7096	1.5418	0.5982	33.85*	15	0.7467
SRCRSP5	3	1.7699	0.2333	0.4424	0.3819	0.7507	0.4636	33.85*	3	0.4350
SRCRSP9	7	5.6075	0.8000	0.8356	0.7975	1.8107	0.0264	33.85*	21	0.8217
Mean	6.4800	4.3993	0.6580	0.7591	0.7105	1.5865	0.1243	-	-	0.7459
S D	1.7588	1.4359	0.2065	0.099	-	0.3173	-	-	-	0.0973

n_a – observed number of alleles, n_e- Effective number of alleles, H_o- Observed heterozygosity, H_e- Expected heterozygosity, PIC- Polymorphism Information Content I- Shannon's Information Index, F_{IS} - Within population inbreeding estimate, F_{ST} –Among sub-population inbreeding estimate, $\chi 2$- Chi- square value, d.f- Degrees of freedom and D- Nei's genetic distance (^{NS}- Not Significant *- significant).

Table 2: Different tests for mutation drift equilibrium in Nellore Sheep

Test	IAM	TPM	SMM
Sign test			
Observed number of loci withHe excess	28	07	12
Expected number of loci with H_e excess	21.07	09.27	18.41
P Value	0.021	0.014	0.191
Standardized differences test			
T_2 value	5.58	3.23	−0.74
value (One tail test for He excess)	0.010	0.046	0.121
Wilcoxon Sign Rank Test			
P value (One tail test for He excess)	0.010	0.091	0.374

IAM=Infinite alleles model, TPM=Two-phase model, SMM=Stepwise mutation model

Conclusion

Hence, even in light of the SMM and qualitative test for mode shift, it is possible to conservatively declare that the sheep population has not departed from the mutation drift equilibrium. This suggests that sheep weren't subjected to a recent genetic bottleneck. However, the heterozygote shortage that unchecked breeding has brought about in the overall population provides cause for uncertainty.

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