Original Research

Studies on Genetic Variability in Nellore Brown Sheep Using Microsatellite Markers

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Abstract

Genetic variation in Nellore Brown sheep was investigated using 24 microsatellite loci. A total of fifty blood samples collected at random from unrelated Nellore Brown sheep maintained by farmers its breeding tract of Andhra Pradesh. A total of 216 alleles were identified in the study. The number of alleles at each locus varied from four (HUJ616) to thirteen (OarCP49) with a mean of 9.0. Allele size ranged from 75(OarCP49) to a maximum of 297 bp (HSC), while allele frequencies ranged from 0.0102 to 0.3980. The number of effective alleles, observed heterozygosity and expected heterozygosity range found to be 3.8757(HUJ616) to 9.6232 (OarCP49), 0.0408(BM1314) to 0.1429 (OarHH35, OarHH41) and 0.7420(HUJ616) to 0.896(OarCP49) with an overall mean of 6.78 ± 1.79, 0.0985 ± 0.025 and 0.8409 ± 0.046 alleles, respectively. All the loci found to be highly polymorphic and the PIC values ranged from 0.6945(HUJ616) to 0.8961(OarCP49) with a mean of 0.8240 ± 0.061. The inbreeding estimates obtained were all positive and varied from 0.8318(OarHH35) to 0.9534 (BM1314).The mean F_{IS} value of 0.8825 ± 0.03 indicated the deficiency of heterozygotes. The Chi-square test revealed that all the 24 loci were showing significant deviation from Hardy-Weinberg Equilibrium.

Key words: Genetic Variability, Microsatellite markers, Nellore Brown Sheep

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Introduction

Sheep biodiversity in India is characterized by high degree of endemism and variations in agro climatic conditions of the different regions has led to the development of various breeds/strains that are well adapted to specific set of environmental conditions. These breeds have generally been named after their place of origin and some based on their prominent characteristics. Sheep contribute greatly to the agrarian economy, especially in areas where crop and dairy farming are not economical and play an important role in the livelihood of a large proportion of small and marginal farmers and landless labourers. Sheep diversity in India is reflected by 42 descript breeds that are well adapted to varied agro ecological conditions of the country. The total sheep population of the country is about 65.06 millions and occupies third position in the world (19th Livestock Census, 2012). The Southern peninsular region has highest percentage of sheep population that are exclusively used for meat production. Nellore sheep is the tallest mutton breed of India and its home tract is Nellore and Prakasam districts of Andhra Pradesh, but widely distributed throughout the state. Three varieties are distinguished phenotypically into Palla (completely white), Jodipi (white withblack patches at specified locations) and Dora/Brown (completely brown). Rams are horned and ewes are almost always polled. Nellore Brown sheep are predominantly present in Kadapa, Kurnool and parts of Anantapur districts of Andhra Pradesh. The ears are long and drooping and most of the animals carry wattles. The overall mean bodyweights of Nellore sheep were 2.95, 13.91, 17.38, 22.39 and 26.61 kg at birth, 3, 6, 9 and12 months of age, respectively (Rani *et al.*, 2014).

Genetic diversity analysis using markers and statistical analysis of the data are the most notable tools for breed characterization and improvement. The measurement of genetic relationships between breeds and genetic heterozygosity within breeds is the task of molecular characterization by using molecular markers (FAO, 2004). Molecular markers can be used for scrutinizing genetic variations at the DNA level between poles apart from populations and individuals and easily assessed by PCR (Radko and Rychlik, 2010). Molecular level studies were carried out in Nellore Jodipi sheep (Vani, 2017 and Surekha, 2015) using ISAG (FAO) recommended microsatellites. But molecular information regarding Nellore Brown sheep is lacking. Therefore, the present investigation was planned with a view to study the genetic variability in Nellore brown sheep with the aid of microsatellite markers.

Materials and Methods

A total of 50 blood samples were collected at random from unrelated Nellore brown Sheep from farmer's flocks predominantly present in Rayalseema region (Kadapa, Kurnool, Anantapur and parts of Chittoor districts) of Andhra Pradesh, India. Two ml of whole blood was collected under aseptic conditions from jugular vein of each animal into a 6ml EDTA coated vaccutainer. The blood samples were processed for DNA extraction by using phenol-chloroform method (Sambrook *et al.*, 1989).The purity and concentration of DNA sample was checked by UV spectrophotometer and 1% agarose gel electrophoresis. A total of twenty four ovine microsatellites were selected based on degree of polymorphism and genomic coverage (FAO, 2004). The selected microsatellite markers complied with the recommendations of the International Society for Animal Genetics (ISAG) and FAO. PCR amplification was carried out in a final reaction volume of 25µl. Reaction mixture contained 50 to 100 ng of genomic DNA, 200 mM of each dNTP, 10 picomoles of each forward and reverse primers, 10X PCR buffer, 15 mM magnesium chloride and 1 unit of Taq DNA

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polymerase. The PCR reaction cycle was carried out with initial denaturation of 5 mins at 94 °C followed by 35 cycles of amplification with annealing temperature ranging from 47°C to 70 °C for different primers and final extension for 10 mins at 72 °C. The amplified PCR products are verified on 2% agarose gel. Number of alleles, microsatellite allele frequencies, effective number of alleles, polymorphism information content (PIC), test of Hardy-Weinberg equilibrium, observed and expected heterozygosity and F-statistics were calculatedby using POPGENE version 1.32 (Yeh *et al*., 1999). The expected heterozygosity (He) of each microsatellite locus was measured (Nei, 1973).

$$H_e = 1 - \Sigma\, Pi^2$$

Where,
Pi = frequency of the i^{th} allele

The observed heterozygosity (H_0) was calculated as the actual percentage of heterozygotes occurring in the sample population.

$$H_0 = \frac{Number\ of\ heterozygous}{Total\ no.of\ samples} \times 100$$

Estimation of Polymorphism Information Content

The polymorphism information content was calculated using the individual frequencies in which the allele occurred at each locus (Nei, 1978).

$$\mathrm{PIC} = 1 - \sum_{i=1}^{j} Pi^2 - 2\sum_{i=j+1}^{j}\sum_{j=1}^{i-1} Pi^2 Pj^2$$

Where

P_i = allele frequency at the homozygous loci
P_iP_j = allele frequency at heterozygous loci

The heterozygosity and PIC values estimated using above formulas and calculated online at *http://www.liv.ac.uk/~kempsj/pic.html*.

Testing for Hardy-Weinberg Equilibrium

The Chi-Square test of goodness of fit was carried out from observed and the expected numbers to check whether the population was in Hardy-Weinberg equilibrium (Falconer and Mackay, 1996) at the loci studied. The bottleneck hypothesis was investigated using BOTTLENECK- 1.2.02 (Cornuet and Luikart, 1996).

Results and Discussion

A total of 216 alleles were observed across twenty four microsatellite loci studied in Nellore Brown sheep and the number of alleles at each locus varied from a minimum of four (HUJ616) to a maximum of thirteen (OarCP49) with a mean of 9.0 ± 1.83 alleles with range of allele size from 75 bp (OarCP49) to 297 bp (HSC) (Table 1).

Table1: Genetic variability measures in Nellore Brown sheep across 24 microsatellite markers

Locus	No. of alleles (na)	No. of effective alleles (ne)	Allele sizes (bp)	Allele frequencies	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Inbreeding estimate (FIS)	Polymorphism information content(PIC)	Hardy-Weinberg equilibrium	
									χ2 value	d.f.
BM757	7	4.2496	174 - 205	0.0102 - 0.3980	0.0816	0.7647	0.8932	0.736	233.059	21
BM827	13	8.2085	145 - 260	0.0102 - 0.1837	0.0612	0.8782	0.9303	0.8781	545.63	78
BM1314	11	8.0706	152 - 203	0.0102 - 0.1837	0.0408	0.8761	0.9534	0.876	528.801	55
BM6506	11	7.577	114 - 214	0.0102 - 0.1939	0.102	0.8711	0.8829	0.871	423.937	55
BM6526	11	9.1293	152 - 201	0.0306 - 0.1429	0.1224	0.8905	0.8625	0.8941	434.769	55
BM8125	6	4.8652	127 - 158	0.0714 - 0.3061	0.102	0.7945	0.8716	0.7647	196.873	15
CSSM31	7	5.3296	155 - 189	0.0102 - 0.2653	0.102	0.8124	0.8744	0.7859	213.097	21
CSSM47	9	6.8797	144 - 181	0.0204 - 0.2245	0.0816	0.8635	0.9045	0.8379	363.474	36
HSC	10	8.0167	196 - 297	0.0408 - 0.1939	0.102	0.8753	0.8834	0.8625	411.603	45
HUJ616	4	3.875	128 - 163	0.2143 - 0.3265	0.102	0.742	0.8625	0.6945	115.064	6
INRA63	10	5.3237	171 - 229	0.0102 - 0.2857	0.1224	0.8122	0.8492	0.7869	416.881	45
MAF214	12	7.445	187 - 289	0.0102 - 0.2041	0.1224	0.8657	0.8586	0.8656	438.665	66
OarCP20	8	7.5266	84 - 96	0.0612 - 0.1633	0.0816	0.8671	0.9059	0.8519	305.302	28
OarCP34	5	4.2885	113 - 128	0.1176 - 0.3431	0.0784	0.7668	0.8977	0.7307	177.354	10
OarCP49	13	9.6232	75 - 128	0.0204 - 0.1735	0.0816	0.8961	0.9089	0.8961	662.41	78
OarFCB48	9	5.7716	135 - 195	0.0204 - 0.2959	0.0816	0.8267	0.9013	0.8061	392.109	36
OarFCB128	12	9.4528	98 - 129	0.0306 - 0.1735	0.0816	0.8942	0.9087	0.8977	521.54	66
OarHH35	9	6.6418	114 - 162	0.0408 - 0.2653	0.1429	0.8494	0.8318	0.8328	341.15	36
OarHH41	9	7.8592	124 -161	0.0510 - 0.1633	0.1429	0.8728	0.8363	0.8591	325.094	36
OarHH47	10	7.732	119 - 158	0.0306 - 0.1939	0.1224	0.8707	0.8594	0.8568	347.854	45
OarHH64	9	7.7452	142 - 186	0.0102 - 0.1531	0.0816	0.8709	0.9063	0.8568	333.651	36
OarJMP8	10	7.622	145 - 198	0.0102 - 0.1837	0.1224	0.8688	0.8591	0.8546	340.882	45
OarJMP29	6	4.831	156 - 188	0.0408 - 0.2959	0.1224	0.793	0.8456	0.7623	175.498	15
OarVH72	5	4.1468	127 - 147	0.0612 - 0.3061	0.0816	0.7589	0.8924	0.7183	154.356	10
Mean	9.0±1.83	6.78±1.79	75-297	0.0102-0.3980	0.0985±0.25	0.8409±0.046	0.8825±0.03	0.8240±0.061		

*** Highly significant (p<0.01)*

The total number of alleles (216) observed in the present study in Nellore Brown sheep were higher than those reported in indigenous sheep breeds by Hepsibha *et al*., 2014 in Coimbatore sheep (134), Surekha., 2015 in Nellore sheep (129) and Vani., 2017 in Nellore sheep (75). While Tolone *et al*., 2012 in Sicilian sheep (259), Ghazy *et al*., 2013 in Egyptian sheep (401), Kunene *et al*., 2014 in Nguni sheep (253), Sassidy-Zaidy *et al*., 2014 in Barbarine sheep (270) and Hoda *et al*., 2014 in Albanian sheep (348) breeds reported higher values than the present findings. The allele size range (75 to 297 bp) measured in the Nellore brown sheep were comparable to allele sizes reported by Musthafa *et al*. (2012) in Najdi sheep, Al-Atiyat *et al*. (2014) in Jordan sheep and Hepsibha *et al*. (2014) in Coimbatore sheep, Hoda *et al*. (2014) in Albanian

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sheep, Sassidy-Zaidy *et al.* (2014) in Barbarine sheep and Surekha (2015) in Nellore Jodipi sheep. The mean number of observed alleles (9.0 ± 1.83) estimated were similar to the earlier reports of Ligda *et al.* (2009) in Greek sheep breeds, Jyotsana *et al.* (2010) in Patanwadi and Marwari breeds, but higher than the findings of Surekha (2015) and Vani (2017) in Nellore Jodipi sheep and lower than the findings of Crispim *et al.* (2014) in Pantaneiro sheep, Sassidy-Zaidy *et al.* (2014) in Barbarine sheep and Hoda *et al.* (2014) in Albanian sheep. The effective number of alleles at each locus provides information on occurrence of predominant alleles and the number of effective alleles across twenty four microsatellite loci studied ranged from 3.88 (HUJ616) to 9.62 (OarCP49). The mean number of effective alleles (6.7821) recorded in the present research were higher than those reported for Kail (3.9471; Ahmed *et al.*, 2014) and Albanian (4.47; Hoda *et al.*, 2014) sheep breeds, whereas, Ghazy *et al.*, 2013 in Rahmani (11.38) and Ossimi (11.81) breeds of Egypt, Crispim *et al.* (2014) in Pantaneiro sheep (10.68), Sassiy – Zaidy *et al.* (2014) in Barbarine sheep (8.06) reported higher mean effective allelic number than the present findings.

The observed heterozygosity ranged from 0.0408 (BM1314) to 0.1429 (OarHH35, OarHH41) with a mean value of 0.0985 ± 0.025, whereas, the expected heterozygosity ranged from 0.7420 (HUJ616) to 0.8961 (OarCP49) with a mean value of 0.8409 ± 0.046 (Table 1).The heterozygosity values observed in the Nellore Brown sheep were within the range of values noticed in Nali, Chokla and Garole breeds (Mukesh *et al.*, 2006). The observed heterozygosity values were less than the expected heterozygosity for all the loci in the present study coincided with the findings of Musthafa *et al.* (2012), Kunene *et al.* (2014), Ahmed *et al.* (2014) and Surekha (2015) in Najdi, Nguni, Kail and Nellore sheep breeds, respectively. The expected heterozygosity values recorded (Table 1) were in accordance with studies of Nanekarani *et al.*, 2011 in Karakul (0.831) sheep. Musthafa *et al.*, 2012 in Najdi sheep (0.67) and Hepsibha *et al.*, 2014 in Coimbatore sheep (0.6255) while, Pandey *et al.*, 2009 recorded lower value for mean expected heterozygosity (0.554) in Bonpala sheep. A substantial difference was seen between observed and expected heterozygosity for all microsatellite markers. Expected heterozygosity is considered to be a better estimate of the genetic variability and a high level of expected heterozygosity obtained in the present study indicated that the population had retained the several alleles which in turn indicated the existence of genetic variability in Nellore Brown sheep.

The inbreeding estimates obtained in this study were all positive and varied from 0.8318 (OarHH35) to 0.9534 (BM1314). The mean F_{IS} value was 0.8825 ± 0.03. A positive values for inbreeding estimates were also found in Nellore sheep (0.1889; Vani, 2017), Pantaneiro sheep (0.12; Crispim *et al.*, 2014) and Nellore (Jodipi) sheep (0.919; Surekha, 2015), whereas, Hepsibha *et al.*, 2014 observed a negative mean FIS (-0.0024 ± 0.07) in Coimbatore sheep. Musthafa *et al.* (2012) for MAF214 (0.5488) in Najdi sheep, Ghazy *et al.* (2013) in Egyptian sheep breeds for HSC (0.244) and OarCP34 (0.313), Hepsibha *et al.* (2014) in Coimbatore sheep breed for loci OarCP49 (0.3084), OarHH64 (0.4733), BM827 (0.1802), OarJMP8

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(0.2485) and INRA63 (0.1959) and Surekha 2015 in Nellore sheep for loci BM827, BM6506, HUJ616 and OarHH41 (1.00) reported fairly high F_{IS} values similar to the present study. The inbreeding coefficient measures the reduction of heterozygosity in an individual because of non- random mating within population and hence F_{IS} values significantly higher and lower than zero reveal inbreeding or outbreeding, respectively. High F_{IS} values noticed in the present study might be due to the consanguinity present in the population and subpopulations. Other reasons might be that the locus is under selection, genetic hitchhiking effect, null alleles and presence of population sub-structure (Wahlund effect). The Polymorphism Information Content (PIC) values for the twenty four loci ranged from 0.6945 (HUJ616) to 0.8961 (OarCP49). The mean PIC value estimated for Nellore Brown sheep in the present study (0.8240 ± 0.061) was in conformity with the mean PIC values estimated by Hoda *et al.* (2014) in Albanian sheep (0.708), Surekha (2015) in Nellore Jodipi sheep (0.819) and Vani (2017) in Nellore sheep (0.7582). Mean PIC values lower than the present study were reported in Najdi (0.71; Musthafa *et al.*, 2012), Coimbatore (0.5851; Hepsibha *et al.*, 2014), Kail (0.60; Ahmed *et al.*, 2014) and Nguni sheep (0.52; Kunene *et al.*, 2014). The PIC values observed by Ghazy *et al*. (2013) in Egyptian sheep breeds ranged from 0.88 to 0.92 with a mean PIC value of 0.903, which was higher than the value obtained in the present study. The PIC values for all the twenty four loci were more than 0.50 indicating high polymorphism across all the loci studied.

The $\chi2$ test revealed that all the twenty four loci were showing significant deviation ($P < 0.01$) from Hardy-Weinberg Equilibrium. Population disequilibria similar to present study was also noticed in 19 loci in Egyptian sheep breeds (Ghazy *et al*., 2013) and 12 out of 24 loci in Coimbatore sheep (Hepsibha *et al*., 2014). This deviation found at all the loci may be attributed to the presence of low frequency null alleles segregating at these loci, high positive F_{IS} (within population inbreeding estimates) values obtained for several loci, selection process which had been progressing in the population and presence of population substructure (Wahlund effect) and systematic and dispersive processes operating in the population.

Sign test revealed little differences between the number of loci observed and expected with heterozygosity excess (Table 2).

Table 2: Statistical tests for mutation drift equilibrium at 24 microsatellite loci under different mutation models in Nellore Brown sheep

		IAM	**TPM**	**SMM**
Sign Test	Expected number of loci with heterozygosity excess	14.45	14.33	14.13
	Observed number of loci with heterozygosity excess	24	23	20
Standardized different Test	T_2 Values	6.505	5.637	4.021
Wilcox Test	One tailed test of heterozygosity excess	0.0000*	0.00000*	0.00002**

IAM- Infinite allele model; TPM- Two Phase Mutation Model; SMM- Step wise Mutation Model ($*p\leq0.05$; $**p\leq0.01$)

Twenty out of 24 studied microsatellite loci had heterozygosity excess, while remaining showed significant ($p \leq 0.01$) heterozygosity deficiency under SMM model. The data set showed mutation drift equilibrium under IAM, TPM, SMM models. Standardized difference test showed non-significant heterozygosity deficiency (T2= 6.505; 5.637; 4.021) in Nellore Brown Sheep under IAM, TPM, SMM models, while Wilcox test revealed that the population had undergone recent bottle neck assuming the SMM. The mode shift test, however revealed mutation drift equilibrium in this sheep population (Fig. 1).

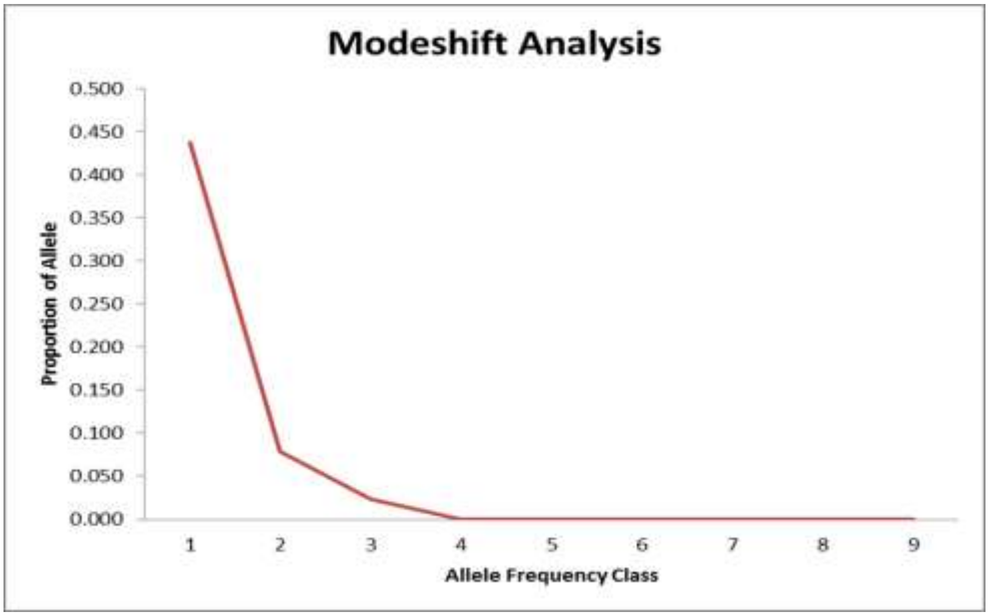

Fig. 1: Mode shift analysis

All above statistical tests used to study mutation drift equilibrium revealed little heterozygosity deficiency in Nellore Brown sheep population under TPM and SMM models. The statistical tests conclude that the Nellore Brown Sheep population had not faced any recent genetic bottle neck.

Conclusion

The results in the present study suggested that there is substantial genetic variation and polymorphism across the studied loci in Nellore sheep. The PIC values more than 0.5 at all the loci indicated the usefulness of these markers in further genetic studies in Indian sheep breeds. Mode shift analysis indicated that the Population is non-bottle necked. Based on population size, even though there is no threat to the population, but high inbreeding coefficient levels in studied population suggestive of adopting effective breeding and management practices for the genetic improvement of Nellore sheep.

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