Original Research

Genetic Diversity Analysis of Macherla Brown Sheep Using Microsatellite Markers

Mohankishore Molabanti, Punyakumari Bhupati*, Suresh Jeepalyam, Bharathi Gutti and Amol Jagannath Talokar

Department of Animal Genetics and Breeding, College of Veterinary Sciences, Tirupati-517502, Andhra Pradesh, INDIA

***Corresponding author:** punya67@yahoo.co.in

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Abstract

A total of 275 alleles were observed for the twenty four marker loci under investigation in the sampled Macherla Brown population. The number of alleles at each locus varied from eight (BM6506, HSC, OarCP34) to eighteen (MAF214) with a mean of 11.458 alleles. Allele size ranged from 69 bp (OarCP20) to 313 bp (MAF214), while allele frequency varied between 0.0102 (HSC, INRA63, MAF214, OarP49 and OarVH72) to 0.2551 (HSC). The number of effective alleles across twenty four microsatellite loci studied ranged from 5.8065 (HSC) to 14.1652 (OarFCB48). The mean number of effective alleles was 9.597 ± 2.454. The observed heterozygosity ranged from 0.0612 (BM8125) to 0.2653 (OarCP34) whereas, the expected heterozygosity varied from 0.8278 (HSC) to 0.9294 (OarFCB48). All the twenty four microsatellite loci (100 percent) were found to be highly polymorphic and the PIC values varied from 0.8052 (HSC) to 0.9295 (OarFCB48) with the mean PIC value of 0.882 ± 0.036. The Chi-square test revealed that all the twenty four loci significantly deviated from Hardy-Weinberg Equilibrium. The inbreeding estimates obtained in this study were all positive and ranged from 0.6915 (OarCP34) to 0.9341 (OarFCB48) with the mean F_{IS} value of 0.876 ± 0.057 indicating the high deficiency of heterozygotes. The bottle neck analysis revealed that population has not undergone any recent reduction. The study revealed that Macherla brown sheep with increasing population trend needs genetic management for the conservation and improvement.

Key words: Bottleneck, Macherla Brown Sheep, Microsatellite Markers, PIC

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Introduction

India is a rich repository of sheep genetic resources with 42 recognized sheep breeds and ranks 3rd in the world with 65.09 million sheep population (19th Livestock Census India., 2012). Indigenous breeds in general demonstrate low production performance when compared to exotic stock. However, they have

better adaptability to pressures of the specific local environment. Adaptive traits that are usually associated with the indigenous breeds include disease resistance, heat tolerance and ability to survive under low input conditions. The Southern peninsular region is endowed with mutton breeds like Nellore, Mandya, Deccani, Hassan, Bellary, Khenguri, Coimbatore, Madras Red, Mecheri etc. Andhra Pradesh possess a sizeable population of non-descript local sheep besides Nellore and Deccani breeds. Macherla Brown, a popular local sheep found predominantly in the villages mainly adjacent to the Krishna river flowing through Prakasam, Guntur, Krishna and Nalgonda districts of Andhra Pradesh and Telangana. The farmers in this area prefer Macherla brown due to their adaptability to adverse climatic conditions. Macherla Brown sheep are characterized by convex head, pendulous ears and black muzzle. Based on coat colour, three phenotypes *i.e* bicolour of brown and white (61.96%), brown (36.46%) and brown and black (14.44%) were noticed. The least squares means for body weights at 2, 4, 6 and 8 teeth of age were 33.79, 39.74, 44.52 and 46.98 kg, respectively (Choudary *et al.*, 2015).

The Food and Agricultural Organization (FAO) of the United Nations has proposed a global programme for the management of genetic resources using molecular methodology for breed characterisation (Bjornstad and Roed, 2001) and this strategy places a strong emphasis on the use of molecular markers to assist the conservation and assessment of endangered breeds and also to determine the genetic status of these breeds**.** Molecular DNA polymorphisms are now the tools of choice for the assessment of genetic diversity among livestock breeds and has potential for discovery of fundamental parameters important in conservation, *viz*., effective population size (Garrigan *et al.,* 2002), bottlenecks (Luikart *et al.,* 1998), population origin (Cornuet *et al.,* 1999) and inbreeding status (Ellegren, 1999; Lynch and Ritland, 1999) of the population. Microsatellites are the most commonly and widely used molecular markers for assessing relatedness in livestock. ISAG/FAO has recommended a list of microsatellite markers for genetic diversity studies in livestock (http://www.fao.org/dad-is).

Phenotypic (Choudary *et al.*, 2015) and cytogenetic characterization (Nityanand *et al.*, 2017) has already been carried out for Macherla Brown sheep. But molecular information is lacking. Hence, the present study is undertaken to characterize Macherla Brown sheep at molecular level with the aid of microsatellite markers.

Materials and Methods

A total of 50 blood samples of genetically unrelated sheep were collected randomly from farmers flocks and the genomic DNA was isolated by using phenol chloroform method (Sambrook *et al.,* 1989). One per cent agarose in 1X TAE buffer (pH 8.0) was used to check the quality of genomic DNA and quantification was done by UV spectrophotometer. The yield of DNA ranged from 58.5 to 851.8 ng/µl with a mean of 170.964 ng/µl and the purity of DNA (OD ratio) ranged from 1.689 to 1.971 with a mean of 1.795. A total

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of twenty four microsatellites were selected based on degree of polymorphism and genomic coverage (FAO, 2004). The selected microsatellite markers complied with the recommendations of the FAO and the International Society for Animal Genetics (ISAG). PCR was carried out in a final reaction volume of 25 µl. Quantity of sample DNA added varied from 2 µl (OarVH72) to 4 µl (BM6526, OarCP34, OarHH41) and a master mix for minimum of ten samples was prepared and aliquoted into each PCR tube to make the final volume of 25 µl. PCR amplification was done on Kyratech thermal cycler. Each 25 µl of PCR reaction mixture consist of 50-100 ng of genomic DNA, 2.5 µl of 10 X Taq buffer, 1 µl of 1.5 mM $Mgcl_2$, 0.5 µl of 200 µM dNTPs, 0.2 µl of 1 unit *Taq* polymerase with 10 picomoles of 1 µl forward and reverse primers. The cycling protocol was 5 min denaturing at 94°C followed by 35 cycles of denaturation at 94°C for 45 seconds annealing at different temperatures (47 to 70°C) depending on primers for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 10 minutes.

The PCR products were resolved on 2% agarose gels stained with ethidium bromide in 1X TAE buffer with 100 bp DNA ladder using as a size standard. The amplified product was visualized as a single compact fluorescent band of expected size under UV transilluminator and photographed with UVi-tech gel documentation system (THERMO SCIENTIFIC ®). The genotypes were scored based on the presence of a single band (homozygotes) or double bands (heterozygotes) on the agarose gel slab. Based on the size of the alleles obtained on the agarose gel electrophoresis, microsatellite allele frequencies, effective number of alleles (N_e), polymorphism information content (PIC), test of Hardy-Weinberg equilibrium, observed (H_o) and expected heterozygosity (H_e) and F-statistics (F_{IS}) were calculated using the POPGENE version 1.3.2 (Yeh *et al*., 1999). Polymorphism information content was calculated using PIC calculator in the google home page.Genetic bottleneck effect was inferred for the population using the qualitative graphical method (mode shift analysis) under the assumption of two phase microsatellite mutation model (TPM), Infinite allele model (IAM), step wise mutation model (SMM) implemented in the programme bottle neck version 1.2.02 (Cornuet and Luikart 1996). This programme is based on the principle that any population that has experienced a recent reduction in its effective population size exhibits a correlative reduction in the allele numbers and gene diversity at polymorphic loci.

Results and Discussion

All the 24 microsatellite loci amplified successfully and were found to be polymorphic. The parameters of genetic variability estimated were detailed in Table 1. Allele diversity: A total of 275 alleles were identified across twenty four microsatellite loci with a mean of 11.458 ± 2.734 alleles.

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Table 1: Genetic structure of Macherla Brown sheep across 24 Microsatellite markers

Locus	No. of alleles (n_a)	No. of effective alleles (n_e)	Allele sizes (bp)	Allele frequencies	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Inbreeding estimate (F_{IS})	Polymorphism information content(P_{IC})	Hardy-Weinberg equilibrium	
									$\chi 2$ value	d.f.
BM1314	11	8.843	157 - 180	0.031 - 0.194	0.102	0.8869	0.8849	0.8869	421.7129**	55
BM6506	8	7.659	175 – 228	0.082 - 0.163	0.102	0.8694	0.8826	0.8548	294.2818**	28
BM6526	14	13.049	154 – 201	0.041 - 0.102	0.102	0.9234	0.8895	0.9234	631.0162**	91
BM757	12	9.379	166 – 216	0.020 - 0.174	0.0816	0.8934	0.9086	0.8934	506.2322**	66
BM8125	11	8.294	134 – 182	0.031 - 0.174	0.0612	0.8794	0.9304	0.8794	484.3134**	55
BM827	11	10.349	165 – 197	0.061– 0.112	0.0612	0.9034	0.9322	0.9035	494.2495**	55
CSSM31	10	8.795	112 – 147	0.020 - 0.143	0.0816	0.8863	0.9079	0.8751	389.8391**	45
CSSM47	10	7.931	146 – 209	0.021 - 0.208	0.1875	0.8739	0.7854	0.8609	304.7294**	45
HSC	8	5.806	203 – 232	0.010 - 0.255	0.0612	0.8278	0.926	0.8052	281.4185**	28
HUJ616	9	7.41	128 – 171	0.02– 0.174	0.0816	0.8651	0.9056	0.8497	319.8519**	36
INRA63	10	8.827	162 – 221	0.010 - 0.14	0.1224	0.8867	0.8619	0.8751	343.0737**	45
MAF214	18	13.839	195 - 313	0.010 – 0.122	0.1429	0.9277	0.846	0.9278	765.3591**	153
OarCP20	14	9.84	69 - 94	0.020 - 0.122	0.1224	0.8984	0.8637	0.8984	638.5662**	91
OarCP34	8	7.135	102 - 148	0.041 - 0.184	0.2653	0.8599	0.6915	0.8435	192.8396**	28
OarCP49	16	12.571	123 - 183	0.010 - 0.112	0.102	0.9204	0.8891	0.9205	725.2924**	120
OarFCB48	11	10.791	130 - 216	0.041 - 0.102	0.0612	0.9073	0.9325	0.9074	485.1454**	55
OarFCB128	15	14.165	133– 153	0.082 - 0.122	0.0612	0.9294	0.9341	0.9295	726.5161**	105
OarHH35	13	11.857	130 - 178	0.051 - 0.112	0.102	0.9157	0.8886	0.9157	547.5290**	78
OarHH41	13	11.516	117 -162	0.031 - 0.122	0.1429	0.9132	0.8436	0.9132	504.4707**	78
OarHH47	9	7.287	126 - 171	0.020 - 0.184	0.0612	0.8628	0.929	0.8472	369.4592**	36
OarHH64	15	12.67	131– 191	0.020 - 0.122	0.1633	0.9211	0.8227	0.9211	550.0499**	105
OarJMP8	10	8.514	114 - 160	0.020 - 0.153	0.1224	0.8825	0.8613	0.8708	387.6712**	45
OarJMP29	10	7.977	142– 181	0.061 - 0.194	0.1623	0.8749	0.8133	0.8614	340.3891**	45
OarVH72	9	5.828	136 - 178	0.010 - 0.235	0.0816	0.8284	0.9015	0.8054	271.5215**	36
Mean	**11.458±2.734**	**9.597±2.454**	**69- 313**	**0.0102-0.2551**	**0.1098 ±0.0494**	**0.8891 ±0.0286**	**0.8763 ±0.0566**	**0.8821 ±0.0362**		

*** Highly significant (p<0.01)*

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The number of alleles at each locus varied from a minimum of eight (BM6506, HSC and OarCP34) to a maximum of eighteen (MAF214). The loci BM1314, BM6506, BM6526, BM757, BM8125, BM827, CSSM31, CSSM47, INRA63, MAF214, OarCP20, OarCP49, OarFCB48, OarFCB128, OarHH35, OarHH41, OarHH64, OarJMP8 and OarJMP29 have amplified more than ten alleles, whereas the rest of loci amplified less than ten alleles. The total number of alleles observed in the present study for Macherla Brown sheep were almost in conformity with the findings of earlier workers (Kunene *et al*., 2014; Sassi-Zaidy *et al*., 2014 and Sheela Manjari *et al.,* 2018). The allele size range (69 to 313) measured in Macherla brown sheep was almost comparable to allele size reported in other sheep breeds *viz.* Jordan sheep (Al-Atiyat *et al*., 2014), Coimbatore sheep(Hepsibha *et al*., 2014) and Nellore Jodipi sheep (Vani *et al.,* 2017). The mean observed number of alleles (11.458) in Macherla Brown Sheep were almost in accordance with the results noticed in Jordan sheep (12.67; Al-Atiyat *et al*., 2014), Balaeric sheep (13.65; Pons *et al*., 2015), while Ligda *et al*. (2009) in Greek sheep (8.34) breeds and Jyotsana *et al*. (2010) in Patanwadi (8.25) and Marwari (9.05) breeds observed lower mean number of alleles. The effective number of alleles varied between 5.806 (HSC) to 14.165 (Oar FCB 48) with an overall mean of 9.597 ±2.454. The mean number of effective alleles (9.597) recorded in the study was in harmony with Sassi-Zaidy *et al*. (2014) in Fat-tailed Barbarian sheep breed (8.66). The effective number of alleles in Macherla Brown sheep is higher than those reported by Kumarasamy *et al*. (2009) in Coimbatore (4.93), Pandey *et al*. (2009) in Bonpala (2.47), Selvam *et al.* (2009) in Madras Red (6.98), Radha *et al.* (2011) in Kilakarsal (3.85) Sheep breeds, whereas, Ghazy *et al*. (2013) reported higher mean effective allelic number in Rahmani (11.38) and Ossimi (11.81) breeds of Egypt with a mean of 14.13.

Earlier literature (Takezahi and Nei, 1996) revealed that microsatellite loci for genetic diversity studies should have more than four alleles in order to reduce the standard error of estimates for genetic distance. Thus the number of alleles for each locus in the present study suggested the suitability of these markers to analyse genetic diversity. The variation in allele number and size in the present study compared to published literature may be attributable to the study of unrelated local populations spread over different geographical areas, which possessed high degree of genetic variation.

Heterozygosity

The observed heterozygosity values in Macherla Brown sheep ranged from 0.0612 (BM8125) to 0.2653 (OarCP34) with a mean value of 0.1098 $\pm$ 0.049, whereas, the expected heterozygosity ranged from 0.8278 (HSC) to 0.9294 (OarFCB48) with a mean value of 0.8891 $\pm$ 0.029 (Table1). The observed genetic variation in Macherla Brown sheep is much lower than the expected heterozygosity and also coincided with the earlier works reported by Kunene *et al*. (2014), Sheela Manjari *et al*. (2018) and Surekha *et al*. (2018) in Nguni, Nellore Brown and Nellore Jodipi sheep breeds, respectively.

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The expected heterozygosity values noticed in this study were almost in harmony with studies of Hepsibha *et al*. (2014) in Coimbatore sheep (0.6255), Al-Atiyat *et al*. (2014) in Jordan sheep(0.678), Das *et al*. (2015) in Turkey sheep breeds (0.734) and Pons *et al*. (2015) in Balearic sheep (0.69). Expected heterozygosity is considered to be a better estimate of the genetic variability, while, the value obtained in the present study indicated that the population had retained several alleles and this correlates with the existence of genetic variability in overall population of Macherla Brown sheep.

Within Population Inbreeding Estimate

The F_{IS} values in the sampled population ranged from 0.6915 (OarCP34) to 0.9341 (OarFCB48). The mean heterozygote deficit (F_{IS}) value observed was 0.8763 ± 0.057 and the F_{IS} values reported are positive across all the twenty four loci studied in Macherla Brown sheep. A positive value of inbreeding estimate was also noticed in Vembur (0.2954; Pramod *et al*., 2009) and Kilakarsal sheep (0.147; Radha *et al*., 2011). Whereas, Hepsibha *et al*. (2014) in Coimbatore sheep (-0.0024) reported negative mean F_{IS} value indicating the absence of inbreeding. Positive F_{IS} value suggested inbreeding to be one of the main causes for lack of heterozygotes in Macherla Brown sheep. The shortage of heterozygotes and excess of homozygotes by the investigated populations might be attributed to a number of factors, viz. assortative mating (sample relatedness), linkage with loci under selection (genetic hitch hicking), population heterogeneity or null alleles (Nei, 1987).The foremost rationale for significant F_{IS} values in these populations seems to be relatedness of few samples under field conditions. From the flock structure of these Macherla Brown sheep it is apparent that rams breed with all the ewes in the flock, as the rams and ewes are housed and grazed together there by no controlled mating is practiced at farmer's level. Generally, few rams breed with all the ewes in the flock and this factor i.e. related individuals used for reproduction might be responsible for high heterozygote deficiency observed in this study.

Polymorphism Information Content

The polymorphism information content (PIC) values ranged from 0.8052 (HSC) to 0.9295 (OarFCB48) with the mean PIC value of 0.8821 ± 0.0362, which was well agreed with the findings of Kumarasamy *et al*. (2009) in Coimbatore sheep (0.8106), Nanekarani *et al*. (2011) in Karakul sheep breed (0.808), Surekha (2015) in Nellore Jodipi sheep (0.819) and Kavitha *et al*. (2015) in Tiruchy Black sheep (0.844), while, a higher PIC values were noticed by Ghazy *et al*. (2013) in Egyptian sheep breeds with a mean PIC value of 0.903. Mean PIC values lower than the present study were recorded in Coimbatore (0.5851; Hepsibha *et al*., 2014), Balochi (0.55), Rakhshani (0.57; Wajid *et al*., 2014), Turkey sheep (0.705; Das *et al*., 2015) and Nellore Jodipi (0.753; Vani *et al*., 2017). The PIC values for all the twenty four microsatellite loci were more than 0.50 indicating the suitability of these markers for the genetic diversity analysis in Macherla

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Brown sheep and also suggested the utility of the selected set of microsatellites in molecular characterization and thereby the genetic variability of the investigated population can be exploited.

Hardy–Weinberg Equilibrium

The χ^2 test values revealed the significant deviation of all the twenty four loci from Hardy-Weinberg Equilibrium which was in agreement with the findings of Selvam *et al.* (2009) in Madras Red, where all the ten loci showed significant departure from HWE, while, Nanekarani *et al*. (2011) in Karakul sheep, found that all the fifteen loci showed significant departure from HWE. Population disequilibria similar to present study was also noticed in 19 out of 27 loci (Kumarasamy *et al.,* 2009) in Coimbatore sheep, 19 loci in Egyptian sheep breeds (Ghazy *et al*., 2013), 12 out of 24 loci in Coimbatore sheep (Hepsibha *et al*., 2014) and only ten among the 25 microsatellites studied in Tiruchy Black sheep (Kavitha *et al*., 2015). This deviation of all the loci of Macherla Brown sheep from HWE may be attributable to the presence of low frequency null alleles segregating at all these loci. High positive F_{IS} (within population inbreeding estimates) values and presence of population substructure (Wahlund effect) could be the other reasons for this deviation obtained at all loci.

The qualitative graphical method of Cornuet and Luikart(1996) was used to visualize the allele frequency spectra in Fig. 1. The microsatellite alleles were classified into ten frequency classes. No mode-shift was detected in the frequency distribution of alleles and normal L-shaped curve was observed. Similar findings were also reported by earlier research workers in Jalauni,Vembur, Nellore jodipi and Nellore Brownbreeds (Arora *et al.,* 2008, Pramod*et al.,* 2009, Surekha*et al.,* 2018, Sheela Manjari*et al.,* 2018).

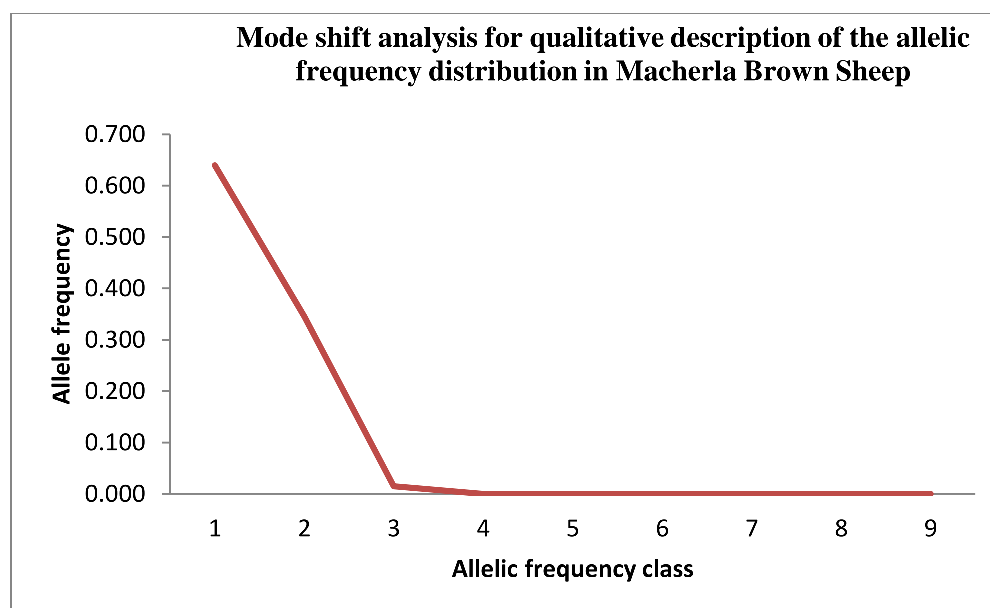

Fig. 1: Mode shift analysis

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The results of bottleneck analysis using three tests viz., sign rank test, standardized difference test and Wilcoxon test in each of three models of mutations namely, infinite allele model (IAM), two phase model (TPM) and step wise mutation model (SMM) are detailed in Table 2. The results of bottle neck analysis showed that the Macherla Brown sheep population has not undergone any recent reduction in the effective population size and remained at mutation drift equilibrium.

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

Model	Infinite Allele Model	Two Phase Model	Step wise Mutation Model
Sign rank test (No. of loci with heterozygosity excess)			
Expected	14.62	14.37	13.99
Observed	24	24	24
Probability	0.00001	0	0
Standardized differences test (T_2 values)	7.489	7.21	6.315
probability	0	0	0.00007
Wilcoxon test (One tail test of heterozygosity excess)	0.00000*	0.00000*	0.00000*

*(*P≤0.05, **P≤0.01)*

The present study revealed that Macherla Brown sheep has sufficient potentiality for genetic improvement with respect to body weights and meat output, by adopting appropriate breeding strategies such as selective breeding, supply of sufficient number of superior rams in the breeding tract, establishing a nucleus flock and implementing an open nucleus breeding system. Creation of awareness among the sheep farmers for better health and managemental practices, exchange of breeding rams between the populations, making timely availability of superior quality rams needs to be ensured for exploitation of genetic diversity and sustainability of this unique germplasm in its home tract. Further, integrating genetic improvement programmes for this Macherla Brown sheep with market oriented production strategies will raise the economy of its rearers and there by ensure its sustainable conservation.

Conclusion

The results in the present study suggested that there is substantial genetic variation and polymorphism across the studied loci in Macherla Brown 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. Based on population size, even though there is no threat to the population, but high inbreeding coefficient levels in studied population was suggestive of adopting effective breeding and management practices for the genetic improvement of Macherla Brown sheep.

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