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Microsatellite- based phylogeny of Indian sheep breeds

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ABSTRACT

Genetic diversity of south Indian meat type breeds was investigated by means of 15 ovine microsatellite markers. All used microsatellites amplified well and exhibited polymorphisms. A wide range of genetic variability was observed as allele varied from 4 to 10 in Madras Red; 4 to 10 in Mecheri; 4 to 12 in Pattanam; and 3 to 12 in Nellore sheep breeds. The mean number of alleles observed in Madras Red, Mecheri, Pattanam and Nellore breeds were 6.5, 6.7, 7.0 and 7.2 respectively. Average observed and expected heterozygosities over the different breeds were 0.23 to 0.87 and 0.347 to 0.827 respectively. The most diverse sheep breed was Nellore breed and the least diverse breed was Madras Red, which had the lowest total number of alleles (n_a=98) and lowest average observed (H_o=0.576) and expected (H_o=0.594) heterozygosities. The multi-locus mean between-population variability (F_{ST}) values indicated that about 5.60% of the total genetic variation was explained by population differences, whereas, the remaining 94.40% is due to differences among individuals within breeds. The values of F_{IT} in the sub population for the most of markers were positive which showed the deficiency of heterozygotes. The D_A genetic distance and F_{ST} distances between pairs of breeds revealed that the lowest distance was between Madras Red and Mecheri and the highest between Mecheri and Nellore breeds. Analysis of individual genotypes provided valuable information for understanding intra and inter-population genetic differences and helps in planning genetic improvement and conservation strategies for further improvement and sustainable utilization.

Key words: Genetic diversity, Microsatellite analysis, Sheep, Tamil Nadu

The sheep in India are distributed in 4 major geographical regions, viz. temperate Himalayan region, northwestern region, southern peninsular region and eastern region (Acharya 1982). Considerable variation exists among sheep populations in terms of size, coat colour, ear, horn pattern and production performance. Genetic markers are used to determine genetic variation between breeds; subsequently relationships among breeds are determined in calculating genetic distance and constructing trees. Microsatellite markers are used to analyse the genetic variation different livestock species (Rout *et al*. 2008, Arora *et al.* 2011a, b, Mahmoudi *et al*. 2012, Mukhongo *et al*. 2014, Mishra *et al*. 2015). Following the guidelines proposed by FAO (1996) under the global project for the measurement of domestic animal diversity (MoDAD), several programmes are in progress in India (Arora and Bhatia 2006, Arora *et al.* 2011a,b) to genetically characterize indigenous sheep breeds by using microsatellite markers, which permit a highly precise dissection of the genetic structure. The genetic relationship of south Indian sheep breeds was previously studied using morphological, physical and biochemical marker. However, no detailed comprehensive study has been made with nuclear molecular/short tandem repeats (STR) markers and covering a wide set of populations from different regions. Therefore, we have carried out the present analysis based on individual genotypes of 15 microsatellite sequences with a view to obtain a deeper insight into relationship within and between breeds.

MATERIALS AND METHODS

Sheep (188) representing four major sheep breeds of south India (viz. Mecheri, Madras Red, Pattanam and Nellore) were sampled from their natural habitat. All the 4 breeds are belonging to meat type breeds and play an important role in livelihood of rural poor people in southern agroclimatic zone of India. About 10 ml of blood samples were collected from each animal's jugular vein using EDTA vacutainers and stored at 4°C until DNA isolation. An effort was made to collect samples from unrelated individuals based on information provided by the farmers. The DNA was isolated according using a modified high salt method (Miller *et al.* 1988). The markers were chosen from the existing ovine genetic maps with an effort to cover all

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chromosomes having high heterozygosity, wide range of alleles and ease of amplification in multiplex polymerase chain reaction (PCR). DNA was amplified by a standard PCR protocol with negative dye PCR premix. The genetic analyser was used for capillary electrophoresis of the PCR product. The estimation of allele size was performed using GeneMapper software ver 4.0. The allele data thus retrieved was subjected to further statistical analysis. The genetic variation within each breed was evaluated and compared. The total number of alleles (n_a) at each locus, the respective allele frequency, observed (H_o) heterozygosity and expected (H_e) heterozygosity for each breed across the locus were calculated using the GenAlEx 6.4 (Peakall and Smouse 2006) software programme. Exact tests for deviations from Hardy-Weinberg Equilibrium (HWE) were performed by GENEPOP package (Raymond and Rousset 1995). The program performs a probability test using a Markov Chain (dememorization 10,000, batches 100, iteration per batch 1000). Analysis of molecular variance (AMOVA), F_{ST} and pair-wise difference were computed using ARLEQUIN ver 3.11 (Excoffier *et al*. 1992). The D_A genetic distance (Nei *et al*. 1983) was calculated and phylogentic trees were estimated using DISPAN program (Ota 1993). The genetic structure and the degree of admixture of four sheep populations were investigated using the Bayesian clustering procedure of STRUCTURE ver 2.3 (Pritchard *et al*. 2000). We carried out 50 independent runs for each K value ranging from 2 to 11. To identify the most probable groups (K) that best fit the data, we used STRUCTURE Harvester (Earl and von Holdt 2012), which implements the Evanno method (Evanno *et al*. 2005). The program CLUMPP ver 1.1 (Jakobsson and Risenberg 2007) was used to align the 50 repetitions of the each K. The output from CLUMPP was then used as an input for DISTRUCT ver 1.1 (Rosenberg 2004), a cluster visualizing software program.

RESULTS AND DISCUSSION

Genetic variation within breeds: Out of the 22 STR markers analysed, 3 failed to amplify in any of the samples, 4 showed monomrophic patterns and the remaining 15 were polymorphic. The total number of alleles and allele size range for each locus are presented in Table 1. Among the polymorphic markers, the ILSTS11, MCM140, ILSTS28 and OarFCM193 loci showed the highest number of alleles (more than 10) and the locus BM1824 exhibited smallest number of alleles. In total, 411 alleles were observed in all the 4 breeds studied. Breed specific alleles were observed at different loci for different breeds with low frequency. The level of variation depicted by the number of alleles at each locus serves as a measure of genetic variability having direct impact on differentiation of breeds within a species. The allele size range observed in the studied population was in agreement with those of other sheep breeds on India and abroad (Kumarasamy *et al*. 2009, Pramod *et al*. 2010, Mukhongo *et al*. 2014). The high genotypic values observed in the present study could be attributed to the high number of allele, which also suggested the existence of heterozygous genotypes in this population. The average gene diversity for different breeds observed in this study ranged from 0.594 to 0.673. Takezaki and Nei (1996) stated that for markers

Table 1. Allele diversity and estimated F_{IS} at different loci in four sheep breeds of Tamil Nadu

Locus	Allele size range	Madras Red		Mecheri		Pattanam		Nellore	
		n_a	F_{IS}	n_a	F_{IS}	n_a	F_{IS}	n_a	F_{IS}
ILSTS11	263–285	10	0.21	8	–0.03	6	0.07	5	–0.08
OarFCB20	87–117	8	–0.05	5	0.09	8	0.08	8	–0.03
OarJMP58	130–168	8	–0.12	9	0.09	9	–0.02	7	0.12
BM1824	170–182	5	0.12	4	0.40	4	–0.04	3	–0.13
OarAE129	110–174	7	0.40	6	–0.05	6	0.32	5	0.29
OarFCB128	95–123	4	–0.12	5	–0.13	4	0.07	5	0.02
ILSTS28	124–172	9	–0.06	9	–0.02	11	0.11	10	0.04
MCM140	167–191	6	0.16	10	0.02	9	0.01	7	–0.03
OarFCB193	94–140	8	–0.05	8	0.07	12	–0.06	12	0.06
BM8125	103–117	5	0.12	8	–0.05	5	0.02	7	–0.15
ILSTS5	179–213	7	–0.10	4	0.36	8	0.14	7	0.01
MAF209	101–125	6	0.09	6	–0.26	6	–0.25	7	0.05
MAF214	181–247	4	–0.19	4	0.04	4	–0.18	9	–0.05
OarCP34	103–121	5	–0.14	6	0.03	6	–0.09	7	–0.11
OarFCB304	136–184	5	–0.01	9	0.13	7	0.07	9	0.05
Mean		6.5	0.018	6.7	0.046	7.0	0.017	7.2	0.003

n_a, observed number of alleles; F_{IS}, estimated inbreeding coefficient.

Table 2. Basic diversity measures and test for Hardy-Weinberg (HW) equilibrium at different loci in 4 sheep breeds of Tamil Nadu

Locus	Madras Red			Mecheri			Pattanam			Nellore		
	Ho	He	HWE	Ho	He	HWE	Ho	He	HWE	Ho	He	HWE
ILSTS11	0.60	0.771	**0.000**	0.76	0.747	0.078	0.69	0.741	0.052	0.72	0.673	0.415
OarFCB20	0.67	0.638	0.493	0.47	0.517	0.063	0.65	0.705	0.142	0.72	0.704	0.711
OarJMP58	0.65	0.581	0.466	0.67	0.744	0.241	0.71	0.699	0.710	0.49	0.558	0.116
BM1824	0.64	0.728	**0.000**	0.42	0.702	**0.000**	0.63	0.605	0.755	0.66	0.587	0.294
OarAE129	0.36	0.596	**0.000**	0.44	0.426	0.640	0.23	0.347	**0.018**	0.36	0.518	**0.016**
OarFCB128	0.60	0.536	0.626	0.73	0.648	0.841	0.54	0.586	0.462	0.70	0.722	0.801
ILSTS28	0.85	0.811	0.280	0.81	0.804	**0.006**	0.71	0.799	0.046	0.78	0.816	0.752
MCM140	0.58	0.698	0.304	0.77	0.794	0.467	0.81	0.827	0.900	0.79	0.765	0.730
OarFCB193	0.56	0.539	0.904	0.70	0.752	0.485	0.87	0.827	0.622	0.77	0.820	0.331
BM8125	0.38	0.429	0.306	0.69	0.659	0.263	0.44	0.450	0.667	0.74	0.649	0.727
ILSTS5	0.48	0.439	**0.014**	0.43	0.672	**0.002**	0.58	0.679	0.205	0.74	0.754	0.471
MAF209	0.50	0.550	0.635	0.69	0.549	0.212	0.73	0.587	0.274	0.64	0.678	0.781
MAF214	0.53	0.450	0.547	0.53	0.561	0.191	0.49	0.418	0.605	0.55	0.532	0.531
OarCP34	0.83	0.733	0.232	0.57	0.588	0.391	0.83	0.768	0.835	0.81	0.731	0.451
OarFCB304	0.42	0.413	0.794	0.55	0.631	0.178	0.38	0.412	0.385	0.55	0.585	0.121
Mean	0.576	0.594		0.615	0.653		0.619	0.630		0.669	0.673	

Ho, observed heterozygosity; He, expected heterozygosity; HWE, P-values of test for HW equilibrium.

to be useful for measuring genetic variation, they should have an average heterozygosity ranging from 0.3 to 0.8 in the populations. This again confirmed that these markers were appropriate for measuring genetic variation.

The observed and expected heterozygosities were relatively similar to those of other domestic sheep breeds investigated earlier (Arora and Bhatia 2004, Sharma *et al*. 2010, Yadav *et al*. 2011, Mukhongo *et al*. 2014). The mean observed heterozygosity values, though lower than the expected values, did not exhibit significant differences when tested using ANOVA (P>0.05), which suggested random mating in Madras Red, Mecheri, Pattanam and Nellore breeds. The high value of expected heterozygosity indicated that the population had retained the presence of several alleles although at lower frequencies. This again implied a substantial amount of genetic variability in Madras Red, Mecheri, Pattanam and Nellore breeds that might be used in planning breeding strategies particularly in populations of small sizes.

The basic diversity measures and test for HWE at different loci in foru sheep breeds are presented in Table 2. The most polymorphisms were detected at the ILSTS28 locus in Madras Red and Mecheri sheep, OarFCB193 locus in Pattanam and OarCP34 locus in Nellore sheep. The most diverse sheep breed was Nellore breed and the least diverse breed was Madras Red, which had the lowest total number of alleles (n_a=98) and lowest observed (H_o=0.576) and average expected (H_o=0.594) heterozygosities. Wilcoxon's signed ranks test indicated that expected heterozygosity was significantly (P<0.05) lower in Madras Red than in other sheep breeds. Deviations from HWE were statistically significant (P<0.05) for 4 loci in Madras Red, three loci in Mecheri and 1 loci each in Pattanam and Nellore sheep breeds.

The F statistics computed for the whole population (Table 3) were 0.056, 0.082 and 0.029 for respectively, F_{ST}, F_{IT} and F_{IS}. Among the 15 loci studied, 5 loci (OarFCB128, BM8125, MAF209, MAF214 and OarCP34) had negative F_{IS} values. An exact test for population differentiation for all pairs of breeds across all loci studied showed that all breeds were significantly (P<0.001) different from each other. Further, an AMOVA analysis was carried out to analyse the variation within and between breeds. To explain how genetic variation is divided within and among the breeds, 5 different AMOVA analyses were performed and groupings were made according to the common branches observed at the constructed phylogenetic trees. The AMOVA revealed that percentage of variation among population was 5.84% and within populations was 94.16%.

The mean F_{IS} value observed was lower than those

Table 3. Global F-statistics at different microsatellite loci in four sheep breeds of Tamil Nadu

Locus	F_{ST}	F_{IT}	F_{IS}
ILSTS11	0.030	0.086	0.058
OarFCB20	0.026	0.049	0.023
OarJMP58	0.116	0.136	0.023
BM1824	0.067	0.161	0.100
OarAE129	0.060	0.306	0.261
OarFCB128	0.059	0.032	–0.028
ILSTS28	0.044	0.066	0.023
MCM140	0.023	0.064	0.043
OarFCB193	0.059	0.071	0.012
BM8125	0.045	0.020	–0.026
ILSTS5	0.175	0.269	0.113
MAF209	0.018	–0.061	–0.081
MAF214	0.052	–0.024	–0.080
OarCP34	0.038	–0.040	–0.081
OarFCB304	0.023	0.090	0.069
Mean	0.056	0.082	0.029

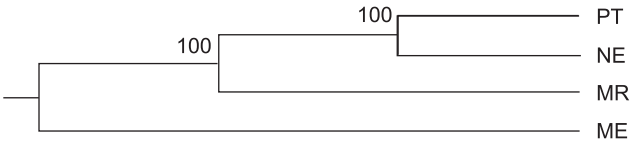


Fig. 1. Neighbour joining tree based on pair-wise Nei's genetic distance among four sheep breeds of Tamil Nadu (PT, Pattanam; NE, Nellore; MR, Madras Red; ME, Mecheri).

reported in earlier studies (Bhatia *et al*. 2008, Pramod *et al*. 2009, Mukhongo *et al*. 2014). The positive F_{IS} value for most of the markers in whole population indicated a general potential risk of inbreeding. This result indicated that non-random mating was performed in sheep breeds studied in the present analysis. The high heterozygote deficiencies could be due to any one or more of the following; segregation of non-amplifying alleles, wahlud effect or inbreeding. Moreover due to uncontrolled mating in India sheep populations at the farmer level, a breeding group most likely comprises a dominant male generally excludes subordinates males, and presumably sire most of the offspring. In addition, the breeding groups will be expected to be inbred with the unequal sex ratio of breeding animals causing inbreeding to accumulate. In case of inbreeding, the deficit affects all or most of the loci in a similar way. In this case farmers do efforts to avoid as much as possible, the breeding between relatives, trying not to use the rams from their own flocks.

The multi-locus F_{ST} values (i.e., mean between-population variability) indicated that about 5.60% of the total genetic variation was explained by population differences, whereas, the remaining 94.40% is due to differences among individuals within breeds. The values of F_{IT} in the subpopulation for the most of markers were positive showing the deficiency of heterozygotes. The multi-locus F_{ST} value observed showed the low differentiation between the subpopulations. This result is similar to those reported for other breeds (Molaei *et al*. 2011, Dashab *et al*. 2011). In general, the genetic analysis of four Indian sheep breeds with 15 microsatellite markers showed higher gene diversity and are in agreement with those of Garole, Nali, Chokla, Muzzafarnagari, Magra and Kilakarisal sheep



Fig. 2. Circular Neighbour joining tree based on pair-wise inter-individual Cavalli-Sforza and Edwards chord distance among four sheep breeds of Tamil Nadu.

Table 4. Pair-wise Nei's genetic distance (upper triangle) and F_{ST} (lower triangle) values among 4 sheep breeds of Tamil Nadu

Breed	Madras Red	Mecheri	Pattanam	Nellore
Madras Red	0	0.098	0.128	0.153
Mecheri	0.065	0	0.132	0.161
Pattanam	0.055	0.060	0	0.058
Nellore	0.077	0.069	0.017	0

Table 5. Proportion of membership coefficient in each of inferred clusters with different runs of STRUCTURE program

Number of K	Breed	Inferred clusters			
		1	2	3	4
K=2	Madras Red	0.122	0.878		
	Mecheri	0.207	0.793		
	Pattanam	0.901	0.099		
	Nellore	0.965	0.035		
K=3	Madras Red	0.098	0.080	0.821	
	Mecheri	0.088	0.239	0.673	
	Pattanam	0.579	0.345	0.076	
	Nellore	0.704	0.268	0.028	
K=4	Madras Red	0.063	0.073	0.062	0.803
	Mecheri	0.069	0.188	0.644	0.098
	Pattanam	0.583	0.313	0.036	0.068
	Nellore	0.695	0.253	0.018	0.033

breeds of India (Arora and Bhatia 2004 2006, Radha *et al.* 2011).

Genetic distance: The D_A genetic distance and F_{ST} distances between pairs of breeds are shown in Table 4. The lowest distance was observed between Madras Red and Mecheri and the highest distance was observed between Mecheri and Nellore breeds (Figs 1, 2). The analysis in STRUCTURE revealed that 4 breeds should be divided in 3 cluster, based on the highest "K value (data not shown) according to Evanno *et al*. (2005). The Pattanam and Nellore breeds were grouped in one cluster and Madras Red and Mecheri were grouped in different clusters (Fig. 3). Proportion of membership coefficient in each of inferred clusters with different runs of STRUCTURE program are presented in Table 5. Based on the consensus phylogenetic tree, populations mainly clustered as per their geographical locations and population identity. From the population structure analysis, the true K value is four (i.e., K=4). At K=4, four breeds were genetically distinct with relatively lower degree of admixture. In the present study at K=2, the similarity across runs were high and consistent, but some variable assignments for breeds were observed for other K values at different runs. Among the 4 breeds, some degree of similarity between Pattanam and Nellore sheep was observed as the common genotype represented by Claret red colour (Fig. 4).

In the phylogenic tree, the south Indian sheep breeds showed a close relationship. The "$_K$ statistics was obviously at a maximum at K=4, which suggested the most probable number of inferred clusters (K=4). The results also supported that the Indian sheep breeds are continuing without introgression of exotic breeds. The data reported here provided a valuable population insight and helped in assessing inter-population dispersal, supporting the idea that analysis of inter-individual relationship is a helpful complement to allele-frequency-based population studies. In addition, the knowledge obtained regarding south Indian sheep breeds as estimated by microsatellite may also be useful as an initial guide in defining objectives for designing future investigation of genetic variation and developing

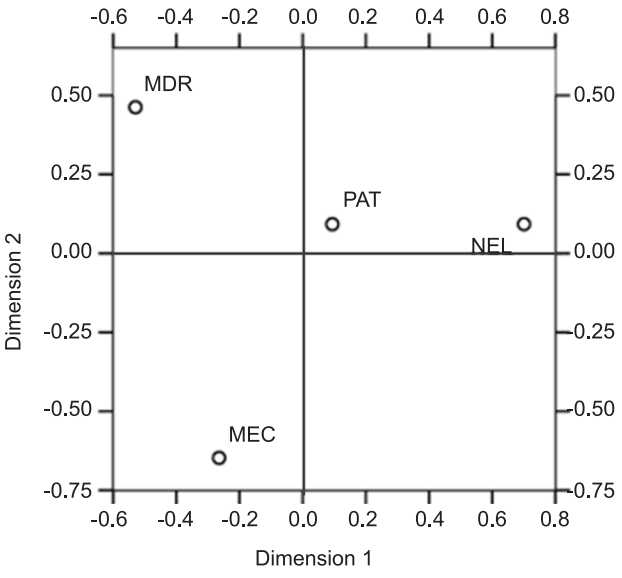


Fig. 3. Multi-dimensional scaling display of pair-wise F_{ST} among different sheep breeds of Tamil Nadu (MDR-Madras Red; MEC-Mecheri; PAT-Pattanam; NEL-Nellore).

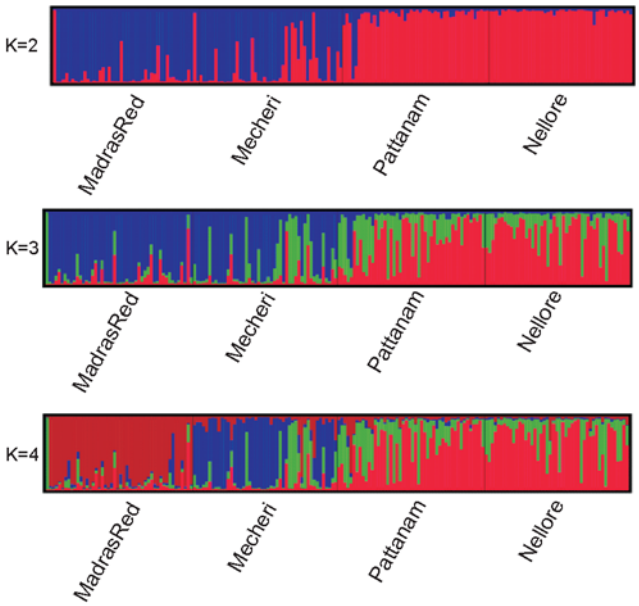


Fig. 4. Graph of the STRUCTURE runs showing membership coefficient of individuals of four sheep breeds in various clusters (K=2 to 4).

conservation strategies. It is also necessary to combine genetic data with geographical positioning and to assess the genetic relationship by geostatitical models in further studies

In conclusion, this analysis showed that microsatellites can be used to classify Indian sheep populations into distinct genetic groups or breeds. Phylogenetic analysis showed the clustering of sheep breeds according to their geographical origin. The breeds studied still maintain genetic distinctness in their natural habitat. Based on the present study, it can be recommended that within breed diversity is actively maintained to enable these extensively unmanaged stocks to adapt to future demands and conditions and there is ample scope for further improvement in its productivity through appropriate breeding strategies.

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