Genetic differentiation between two dairy type river buffalo breeds (*Bubalus bubalis*) of North India using microsatellite markers

P Kathiravan, RS Kataria, SS Bulandi, DK Sadana and BP Mishra

National Bureau of Animal Genetic Resources, Karnal - 132001, Haryana, India

ABSTRACT

The present study was undertaken with the objective of investigating the genetic structure of Nili-Ravi and Murrah buffaloes, the two important dairy type river buffalo breeds of North India. A total of 95 animals representing Nili-Ravi and Murrah buffaloes were analyzed using 24 heterologous cattle microsateliite markers which revealed an average of 5.28 and 5.16 alleles per locus respectively. The average inbreeding coefficient (F_{IS}) was substantially high in both Nili-Ravi and Murrah buffaloes, with significant deficit of heterozygotes viz. 18.8% and 22.8% respectively. The test for Hardy-Weinberg equilibrium showed significant deviations at most of the loci in both the populations except 9 in Nili-Ravi and 8 in Murrah buffaloes respectively. The mean multilocus F_{ST} value (0.124) suggested significant ($P<0.01$) degree of breed differentiation. The Neighbour-Joining tree constructed from allele sharing distance measures among individual animals showed two distinct clusters of the two breeds. Principal component analysis supported this genetic differentiation as the scatter diagram revealed distinct clustering of individuals of these two breeds. Bayesian cluster analysis also revealed a similar type of genetic structure, with the proportion of membership coefficient in each of the two inferred populations being more than 98% from either of the two source population respectively. The genetic distinctness of these two north Indian dairy type buffalo breeds as revealed by microsatellite analysis may have significant impact on issues concerning conservation and biodiversity.

Key words: Buffalo, Genetic structure, Murrah, Nili-Ravi, microsatellite markers

INTRODUCTION

Livestock has been an integral component of traditional agriculture since time immemorial. In India, buffaloes play pivotal role in livestock production through contributions in terms of milk, meat, hide and draft power for agricultural operations. India is habitat of the best riverine breeds of buffalo in the world. Out of the 10 well defined buffalo breeds, Nili-Ravi and Murrah are considered to be the best dairy types in the Indian subcontinent.

Up to the early 1930s Nili and Ravi buffaloes were considered as varieties of Murrah which differed little except in geographical location (Olver, 1938). However, they were shown as different breeds in the late 1930s as listed in the second and third All India cattle shows. The original home tract of Nili-Ravi buffaloes was the erstwhile undivided Punjab and the animals of this breed can now be located in Ferozepur, Amritsar and Gurdaspur districts in the state of Punjab. Rohtak, Jind, Hisar, Jhajhar, Fatehbad and Gurgaon districts of Haryana state form the original tract of Murrah buffaloes. Nili-Ravi buffaloes are more similar to Murrah, both being large sized breeds and having many characteristics in common except in terms of certain morphological features like five white markings called "*Panch Kalyani*" and "*Wall eyes*". Nili-Ravi buffaloes are widely distributed in neighbouring Pakistan and in the past had traveled to other countries being primarily used as improver breed for milk production especially in China and Philippines. The Murrah breed is well renowned as global breed and close to Nili-Ravi in milk performance traits. However, the genetic relationship between these two important dairy breeds of buffalo has not been precisely delineated.

Investigation of genetic relationship among populations can be done using highly polymorphic genetic markers like microsatellites. They provide lot of genetic information permitting alternative approaches to evaluate genetic differentiation like analysis of inter-individual distances based on the proportion of shared alleles and the assignment of individuals to populations. Different assignment procedures have been developed including those reported by Paetkau et al. (1995), Rannala and Mountain (1997) and Pritchard et al. (2000).

Studies on genetic structure of Nili-Ravi buffalo population and their relationship with Murrah buffaloes assume significance from conservation point of view as well as in designing optimal breeding strategies for their genetic improvement. Moreover, the Nili-Ravi population is declining fast in its breeding tract (Punjab state of India) with an estimated population size of 0.2 million (Vij and Tantia, 2005). The present study was undertaken with the objectives of evaluating the genetic diversity within Nili-Ravi buffalo population and to estimate its relationship with that of Murrah buffaloes.

MATERIAL AND METHODS

Blood samples and microsatellite genotyping: Blood samples were collected from 95 unrelated individuals belonging to Nili-Ravi (47) and Murrah (48) breeds of buffaloes. Although no pedigree records were available, care was taken to ensure unrelatedness among the samples by selecting animals from distinct villages and also by detailed interview of the farmers. Genomic DNA was isolated from blood samples following standard procedure of SDS and proteinase K method (Sambrook and Russell,

2001). A total of 24 microsatellite markers were analyzed on all the investigated animals. All the selected markers were originally identified in cattle and evaluated for diversity analysis in buffaloes (Navani et al. 2002).

PCR amplification of microsatellite loci was performed utilizing 50-100ng genomic DNA in a 25µl reaction volume using PTC-200 (MJ Research Inc., MA, USA). The PCR program comprised of initial denaturation at 94°C for 2 minutes, 30 cycles of 94°C for 1 minute, precise annealing temperature of primers for 1 minute and 72°C for 1 minute followed by a final extension at 72°C for 5 minutes. The PCR products were resolved on 6% denaturing polyacrylamide gels (Sequi GT system, Bio-Rad, USA) and sized using a 10bp ladder (Invitrogen, Life Technologies, CA, USA) as a standard. Size of the alleles was estimated online using INCHWORM program (http://www.molecularworkshop.com/ program/ inchworm.html).

Table 1 List of microsatellite loci analyzed, allele size range, observed and effective number of alleles in Nili-Ravi and Murrah buffaloes

Locus	Allele Size Range	Nili-Ravi		Murrah	
		Observed number of alleles	Effective number of alleles	Observed number of alleles	Effective number of alleles
CSRM 060	94-136	7	4.35	8	4.37
ILSTS 026	138-152	5	3.05	5	2.63
HEL 013	167-191	7	4.33	8	3.86
ILSTS 030	152-168	4	2.68	5	3.72
ILSTS 033	140-158	6	3.21	4	2.43
ILSTS 017	110-122	5	3.28	5	2.85
ILSTS 019	172-182	3	1.66	3	1.18
ILSTS 045	142-152	5	3.01	3	1.23
ILSTS 034	144-154	4	2.54	4	2.30
ILSTS 058	120-150	7	4.80	8	6.21
ILSTS 056	144-194	5	1.58	5	2.13
ILSTS 068	113-125	5	4.51	5	3.80
CSSM 066	172-188	6	5.26	5	3.76
ILSTS 036	125-169	5	4.03	5	3.61
ILSTS 095	199-219	5	2.42	6	2.51
ILSTS 029	160-170	4	1.34	5	1.86
ILSTS 028	145-183	7	4.78	6	3.37
ILSTS 025	116-132	5	2.49	5	2.99
ILSTS 052	144-182	10	3.67	8	5.40
ILSTS 031	176-188	4	1.61	3	1.54
ILSTS 073	170-174	2	1.51	2	1.47
BM 1818	252-276	5	1.58	4	1.92
ILSTS 061	139-165	7	4.35	8	3.83
ILSTS 008	164-204	5	3.84	5	4.47
Mean	-	5.28	3.16	5.16	3.05

Statistical analysis: Allele frequency, observed and effective number of alleles, observed and expected heterozygosity estimates were computed after Nei (1973) using POPGENE software (Yeh et al. 1999). Ewens-Watterson neutrality test based on Manly (1985) and departure from Hardy Weinberg equilibrium based on exact test were also performed using the same program. Heterozygote deficiencies (F_{IS}) and F_{ST} (Weir and Cockerham, 1984) were computed using FSTAT 2.9.3.2 software (Goudet, 2002). Genetic distances among populations based on Nei et al. (1983), Nei's (1972) standard genetic distance, Cavalli-Sforza and Edwards Chord distance and allele sharing inter-individual distance were estimated using MICROSATELLITE ANALYZER (MSA) version 3.15 (Dieriger and Schlotterer, 2003). Pair wise distance matrix based on the proportion of shared alleles with individuals as taxonomic unit was utilized to construct neighbour-joining tree using PHYLIP version 3.5 (Felsenstein, 1993) and the tree was visualized using TREEVIEW version 1.6.6 software. Pair-wise chord

distances between individual animals were utilized to perform principal component analysis using SPSS version 13.0. A scatter gram of the first three largest principal component scores was examined to visualize the geometric relationship between individual animals. Breed differentiation was further investigated using Bayesian clustering approach implemented in STRUCTURE program (Pritchard et al. 2000). Individual animals were assigned to different clusters based on their multilocus genotypes. Admixture model was used with a burn in period of 1000000 iterations and 100000 Markov Chain Monte Carlo (MCMC) repetitions to calculate the probable number of genetic clusters.

RESULTS

A total of 95 animals representing Nili-Ravi and Murrah buffaloes were analyzed using 24 microsateliite markers. The allele size range, observed and effective number of alleles at different microsatellite loci analyzed are presented in Table 1. A total of 128 and 125 alleles were observed across 24 loci with a mean of 5.28 and 5.16 in Nili-Ravi and Murrah buffaloes respectively. Allelic polymorphism varied between 2 (ILTSTS 073) to 10 (ILSTS 052) in Nili-Ravi and between 2 (ILTSTS 073) to 8 (CSRM 060, HEL 013, ILSTS 058, ILSTS 052, ILSTS 061) in Murrah buffaloes respectively. The mean effective number of alleles was lesser than observed number of alleles in both the breeds with 3.16 and 3.05 in Nili-Ravi and Murrah respectively.

Different measures of genetic variation like observed and expected heterozygosity, Nei's expected heterozygosity and heterozygote deficiency are presented in Table 2. Observed heterozygosity varied between 0.174 (ILSTS 029) to 0.891 (ILSTS 061) and 0.046 (ILSTS 045) to 0.750 (ILSTS 052) in Nili-Ravi and Murrah buffaloes with a mean of 0.506 and 0.479 respectively. The mean expected heterozygosity was 0.633 and 0.613 in Nili-Ravi and Murrah respectively. The average inbreeding coefficient (F_{IS}) was substantially high in both Nili-Ravi and Murrah buffaloes, with significant deficit of heterozygotes viz. 18.8% and 22.8% respectively. The test for Hardy-Weinberg equilibrium showed significant deviations at most of the loci in both the populations except 9 in Nili-Ravi and 8 in Murrah buffaloes respectively (Table 4).

Table 2 Summary of heterozygosity and F_{IS} at 24 different microsatellite loci in Nili- Ravi and Murrah buffaloes

Locus	Heterozygosity (Nili-Ravi)			Heterozygosity (Murrah)			F_{IS}	
	Ho*	He*	Nei's He	Ho*	He*	Nei's He	Nili-Ravi	Murrah
CSRM 060	0.448	0.783	0.770	0.619	0.781	0.772	0.418	0.198
ILSTS 026	0.583	0.682	0.672	0.429	0.627	0.619	0.132	0.308
HEL 013	0.841	0.778	0.769	0.619	0.750	0.741	-0.093	0.164
ILSTS 030	0.378	0.634	0.627	0.435	0.739	0.731	0.397	0.405
ILSTS 033	0.326	0.696	0.689	0.405	0.595	0.588	0.526	0.312
ILSTS 017	0.587	0.703	0.695	0.341	0.657	0.649	0.116	0.475
ILSTS 019	0.456	0.401	0.396	0.167	0.158	0.156	-0.152	-0.067
ILSTS 045	0.617	0.675	0.668	0.046	0.191	0.189	0.076	0.759
ILSTS 034	0.739	0.614	0.607	0.596	0.572	0.566	-0.218	-0.053
ILSTS 058	0.511	0.801	0.792	0.489	0.848	0.839	0.355	0.417
ILSTS 056	0.426	0.373	0.369	0.511	0.536	0.530	-0.154	0.036
ILSTS 068	0.362	0.787	0.778	0.619	0.746	0.737	0.535	0.160
CSSM 066	0.851	0.819	0.810	0.578	0.742	0.734	-0.050	0.213
ILSTS 036	0.571	0.761	0.752	0.583	0.733	0.723	0.240	0.193
ILSTS 095	0.239	0.593	0.587	0.091	0.609	0.602	0.593	0.849
ILSTS 029	0.174	0.257	0.255	0.300	0.469	0.463	0.317	0.352
ILSTS 028	0.522	0.810	0.710	0.691	0.711	0.703	0.340	0.017
ILSTS 025	0.364	0.608	0.598	0.643	0.674	0.666	0.392	0.034
ILSTS 052	0.596	0.736	0.728	0.750	0.823	0.815	0.181	0.079
ILSTS 031	0.326	0.365	0.380	0.356	0.354	0.350	0.143	-0.015
ILSTS 073	0.432	0.343	0.339	0.313	0.321	0.318	-0.275	0.016
BM 1818	0.178	0.370	0.366	0.369	0.485	0.479	0.514	0.229
ILSTS 061	0.891	0.779	0.770	0.689	0.747	0.739	-0.157	0.068
ILSTS 008	0.500	0.748	0.740	0.535	0.785	0.776	0.324	0.311
Mean	0.506	0.633	0.625	0.479	0.613	0.606	0.188	0.228

* Ho = Observed Heterozygosity; * He = Expected Heterozygosity

Results of F-statistics in the two buffalo breeds analyzed are presented in Table 3. Mean estimates of F-statistics obtained from jackknifing over all loci were 0.307, 0.124 and 0.207 for F (F_{IT}), f (F_{IS}) and θ (F_{ST}) respectively. The global analysis indicated an overall heterozygote deficiency of 20.7%. The mean multilocus F_{ST} value suggested a highly significant (P<0.01) degree of breed differentiation with 87.6% of the genetic variation explained by between individuals within breed and 12.4% of the variation resulting from between breeds.

Six different methods were employed to estimate genetic distance between the two studied buffalo breeds. Nei's distance (Da), Nei's standard genetic distance (Ds), Cavalli-Sforza and Edwards chord distance (Dc) and allele sharing distance (Dps) were 0.176, 0.277, 0.336 and 0.366, respectively. All these distance based methods showed considerable level of genetic differentiation between Nili-Ravi and Murrah buffalo breeds. Principal component analysis supported this genetic structure as the scatter diagram revealed distinct clustering of individuals of these two breeds (Figure 2).

Table 3. Global F-Statistics for each of 24 microsatellite loci analyzed across Nili-Ravi and Murrah buffaloes

Locus	Fit (F)	Fst (θ)	Fis (f)	Locus	Fit (F)	Fst (θ)	Fis (f)
CSRM 060	0.305	0.006	0.300	ILSTS 036	0.227	-0.006	0.231
ILSTS 026	0.241	0.003	0.239	ILSTS 095	0.823	0.358	0.725
HEL 013	0.036	-0.006	0.042	ILSTS 029	0.363	0.019	0.351
ILSTS 030	0.531	0.198	0.415	ILSTS 028	0.292	0.108	0.207
ILSTS 033	0.569	0.214	0.451	ILSTS 025	0.215	0.024	0.196
ILSTS 017	0.316	0.000	0.316	ILSTS 052	0.207	0.079	0.138
ILSTS 019	0.601	0.627	-0.067	ILSTS 031	0.070	-0.010	-0.079
ILSTS 045	0.442	0.268	0.238	ILSTS 073	-0.127	-0.009	-0.117
ILSTS 034	0.222	0.296	-0.105	BM 1818	0.665	0.476	0.361
ILSTS 058	0.445	0.076	0.399	ILSTS 061	-0.024	0.011	-0.036
ILSTS 056	0.007	0.029	-0.023	ILSTS 008	0.360	0.051	0.326
ILSTS 068	0.415	0.069	0.371	Mean	0.307	0.124	0.207
CSSM 066	0.161	0.088	0.079				

Further, to confirm the genetic distinctness of these breeds, Bayesian clustering analysis was performed to assign the individuals to different clusters using STRUCTURE program. The program STRUCTURE was initially run with a number of expected populations ranging from K=1 to K=4, so as to choose the appropriate value of K which suits best to the whole dataset. Several independent runs for each 'K' were performed to verify that the estimates were consistent across runs. The best value of lnPr (X/K) was obtained for K=2 (-5366.4). The proportion of membership coefficient of the two sampled breeds in each of the two clusters is depicted in Figure 3. The first cluster had contributions of 99.2% from Murrah breed while the second cluster had 98.7% contribution from Nili-Ravi buffaloes.

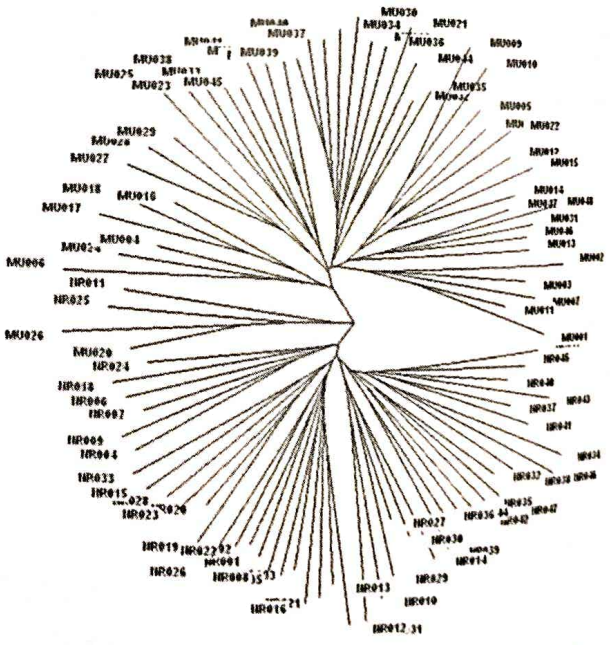

Figure 1. Phylogenetic tree constructed based on pair-wise allele sharing distances between individuals using NJ procedure (MU – Murrah; NR – Nili-Ravi)

DISCUSSION

The present study reports the first comprehensive within breed diversity analysis of Nili-Ravi buffaloes and their genetic relationship with the best dairy type buffalo of the region, Murrah using microsatellite markers. The genetic analysis of 24 microsatellite loci showed that Nili-Ravi buffaloes have moderate level of diversity as reflected by the average number of observed alleles (5.28) and average observed heterozygosity (0.506). However, these estimates are comparatively lower than that of eight other Indian breeds of buffaloes (Kumar, et al. 2006) using a different set of microsatellite markers.

Table 4. Test for Hardy-Weinberg equilibrium at 24 microsatellite loci in Nili-Ravi and Murrah buffaloes

Locus	Nili-Ravi			Murrah		
	DF	Chi Square	P-value	DF	Chi Square	P-value
CSRM 060	21	102.90	0.000	28	114.75	0.000
ILSTS 026	10	12.90	0.230	10	113.20	0.000
HEL 013	21	23.70	0.308	28	76.76	0.000
ILSTS 030	6	38.29	0.000	10	35.36	0.000
ILSTS 033	15	54.72	0.000	6	16.72	0.010
ILSTS 017	10	21.11	0.020	10	73.99	0.000
ILSTS 019	3	2.29	0.515	3	0.34	0.952
ILSTS 045	10	9.30	0.504	3	52.87	0.000
ILSTS 034	6	7.71	0.260	6	5.20	0.518
ILSTS 058	21	81.60	0.000	28	79.73	0.000
ILSTS 056	10	3.24	0.975	10	3.40	0.970
ILSTS 068	10	61.92	0.000	10	20.83	0.022
CSSM 066	15	28.95	0.016	10	19.01	0.040
ILSTS 036	10	46.32	0.000	10	76.57	0.000
ILSTS 095	10	44.84	0.000	15	209.21	0.000
ILSTS 029	6	22.64	0.001	10	31.62	0.000
ILSTS 028	21	55.45	0.000	15	3.59	0.999
ILSTS 025	10	79.19	0.000	10	12.05	0.281
ILSTS 052	45	114.20	0.000	28	41.85	0.045
ILSTS 031	6	24.45	0.000	3	0.17	0.982
ILSTS 073	1	3.13	0.077	1	0.03	0.855
BM 1818	10	56.79	0.000	6	11.86	0.065
ILSTS 061	21	18.96	0.588	28	34.04	0.199
ILSTS 008	10	35.83	0.000	10	47.95	0.000

Considerable level of heterozygote deficiency was noticed in Nili-Ravi and Murrah buffaloes. The global analysis on all the 95 individuals also revealed a heterozygote deficiency of 20.7%. This was further realized by deviations of both the population from Hardy Weinberg equilibrium at many of the studied loci. Gametic disequilibria could be triggered by diverse sources including presence of null alleles, Wahlund effect, physical linkage and epistatic selection (Callen et al. 1993; Pemberton et al. 1995). However, most of the investigated loci were found to be neutral as revealed by Ewens –Watterson neutrality test except ILSTS 058 and ILSTS 008 in Murrah and ILSTS 068, CSSM 066, ILSTS 036 and ILSTS 008 in Nili-Ravi buffaloes. Also, the exact test for linkage disequilibrium did not reveal significant P-values suggesting independent assortment of the microsatellite loci evaluated.

Both the breeds of buffaloes analyzed in this study had been traditionally selected for high milk production in the breeding region. With the advent of A.I., the use of semen from intensively selected bulls generally favours the presence of sire lines leading to increased consanguinity. This is true especially in case of Murrah buffaloes which showed higher heterozygote deficiency (22.8%) than that of Nili-Ravi buffaloes. However, other causes such as segregation of low frequency null alleles and possible relatedness of few samples (although care was taken to collect samples from unrelated individuals) with the absence of pedigree records in the field might have played their part for the high observed heterozygote deficiency.

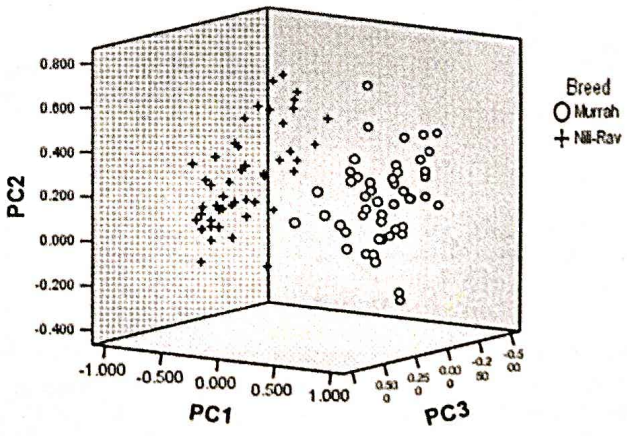

Figure 2. Scattergram showing relative position of individuals of Murrah and Nili-Ravi buffaloes as defined by the three largest principal component scores

Estimation of genetic differentiation (mean F_{ST} value) indicates 12.4% of the variation corresponds to that of between breeds. This is higher than the range of values (0.75% to 6%) reported among other Indian buffalo breeds (Kumar et al. 2006). However, this is comparable to the estimates in diversity studies of other livestock species; 13% in pigs (Martinez et al. 2000); 7-11% in European cattle (MacHugh et al. 1998; Kantanen et al. 2000; Canon et al. 2001). Genetic distance estimations based on different methods revealed considerable differentiation between Nili-Ravi and Murrah breeds. Nei's distance (Da) and Nei's standard genetic distance were estimated to be 0.176 and 0.277 respectively. These values are comparatively higher than the value (0.109) reported by Sodhi et al. (2006) between these two breeds of buffaloes derived by RAPD marker analysis. The Neighbour-Joining tree constructed from allele sharing distance measures among individual animals reflected the genetic distinctness between these breeds (Figure 1). Two distinct clusters were observed with all the individuals except two from each breed coincided with their respective source population. This clustering was consistent with the results of principal component analysis. Visualization of the geometric relationship between individual animals through scatter diagram using the first three principal component scores revealed distinct clustering of the two breeds of buffaloes (Figure 2). The first three principal components explained 39.4%, 8% and 4.8% of the total variance respectively and together explained 52.2% of the total variance.

Bayesian clustering analysis further supported these results with the proportion of membership coefficient in each of the two inferred populations being more than 98% from either of the two source populations respectively. Since STRUCTURE performance is based on the assumption

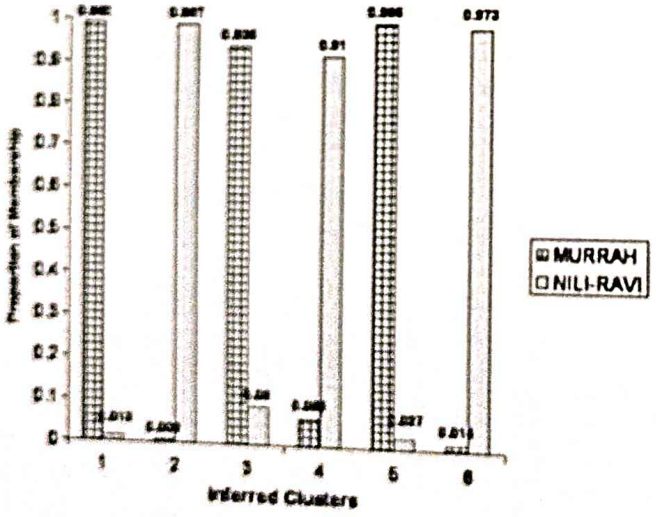

Figure 3. Proportion of membership of Murrah and Nili-Ravi in each of the two inferred clusters (1, 2 – Based on complete data set with 24 loci; 3, 4 – Based on data of 5 Loci in HWE and 5, 6 – Based on data of 19 Loci not in HWE) obtained from STRUCTURE

of HWE, reliability of the observed results was checked by re-running the program twice, firstly using the microsatellite markers (ILSTS 019, ILSTS 034, ILSTS 056, ILSTS 073 and ILSTS 061) which were in HWE in both the populations and again with the remaining markers. However, the results of the analysis revealed that deviations from HWE at meta-population level did not substantially affect the results. The additional runs gave the best values of lnPr (X/K) for K=2 (-923.3 and -4468.7) and the membership pattern did not deviate much from the results of the complete data set (Figure 2).

The present study thus reveals the genetic structure of these two important dairy breeds of India with considerable genetic differentiation between them. The genetic distinctness of these two closely located breeds may have significant impact on issues concerning conservation and biodiversity. This is particularly important in the context of declining trend found in the population of Nili-Ravi breed. Considering the significance of this breed owing to its milk production performance under tropical conditions and typical phenotypic characteristics, it would be appropriate to manage the Nili-Ravi population separately to preserve its genetic uniqueness. Well-planned conservation and improvement effort for this breed is of prime importance in this regard.

ACKNOWLEDGEMENT

We thank the Director, NBAGR, Karnal for providing facilities and ICAR for financial support in carrying out this research work.

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