



Short Tandem Repeat (STR) based assessment of genetic diversity of Alambadi– A draught cattle breed of Tamil Nadu

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ABSTRACT

Alambadi is one of the five indigenous draught type cattle breeds of Tamil Nadu. The present study was undertaken to establish baseline genetic diversity information and evaluate its genetic relationship with Bargur cattle. The results suggested moderate levels of allelic diversity and observed heterozygosity with an overall mean of 6.52 and 0.666 respectively. Estimates of F_{IS} showed significant heterozygosity deficit (0.056) indicating relatively higher levels of inbreeding in Alambadi cattle. The test for Hardy-Weinberg equilibrium revealed 11.1% (3 out of 27) of the investigated loci showing significant deviations due to heterozygosity deficit. Estimation of global F statistics revealed low genetic differentiation between Alambadi and Bargur cattle. The global F_{ST} indicated only 3% of the total variation being explained by between breed differences, while the remaining 97% was explained by within breed variability. Principal components analysis revealed separate clustering of Alambadi and Bargur cattle, although admixture was observed among few animals from both the breeds. The test for mutation drift equilibrium revealed no evidences for the occurrence of genetic bottleneck in Alambadi and Bargur cattle in the recent past. Considering the rapid decline in the population of Alambadi cattle, the results of the present study is expected to help planning the strategy for genetic conservation and breed improvement.

Key words: Diversity, Microsatellite, Mutation Drift Equilibrium, Principal Components,

India, one among the 21 mega diversity hotspots in the world, which harbours a rich domestic animal biodiversity. Indigenous cattle in India belong to the humped *Bos indicus* with high levels of adaptation to prevailing ecological conditions and local farming practices. As per the second report on the Status of World's Animal Genetic Resources (FAO, 2015) there are 60 local breeds, 8 regional trans-boundary breeds and 7 international trans-boundary breeds of cattle in India. Of these, only 41 have been registered by National Bureau of Animal Genetic Resources, the nodal agency for the registration of livestock breeds in India. South India possesses 14 registered indigenous cattle breeds (<http://www.nbagr.res.in/regcat.html>).

In the southern state of Tamil Nadu, there are 4 well

characterized cattle breeds (Kangayam, Umblachery, Bargur and Pulikulam) and one yet to be characterized draught breed called Alambadi. Alambadi has its origin in Dharmapuri district of Tamil Nadu state and is mostly reared in the hilly regions of the Western Ghats. The animals are medium sized with dark grey coat colour and white markings on forehead, tail, and limbs. Bulls can reach up to 350–380 kg body weight at maturity while cows reach 300 kg. Alambadi cattle are poor milkers, but they are known for their draught ability, particularly ploughing and pulling cart. Although they are not fast trotters, Alambadi bullocks are active, hardy and suitable for heavy draught. These cattle mostly survive by grazing in forest regions and are thought to have descended from Hallikar cattle of Mysore. However, information on the baseline phenotypic and genetic characteristics of Alambadi cattle is very limited. With increased mechanization and emphasis on milk production, the population of purebred Alambadi cattle is decreasing and genetic dilution/erosion is occurring at a faster rate.

Investigation of molecular genetic diversity is a valuable complement to evaluate phenotypes and production systems. It provides insights into breed history, guides breed development and helps in conservation decision making (Ajmone-Marsan *et al.* 2014). Molecular tools such as autosomal microsatellite DNA markers have been helpful

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for estimation of population diversity, genetic distance, differentiation and admixture (Pham *et al.* 2013; Sharma *et al.* 2015). Hence, the present study was undertaken with the following objectives: (i) to evaluate genetic variability within Alambadi cattle using multi locus genotype data (ii) to assess its genetic relationship with Bargur cattle and (iii) to test mutation drift equilibrium and identify genetic bottleneck if any.

MATERIALS AND METHODS

Sampling, DNA extraction and Genotyping: Blood samples were collected from a total of 78 unrelated cattle belonging to Alambadi (n=30) and Bargur (n=49) breeds from different regions of their respective native tract following the guidelines of MoDAD (Measurement of Domestic Animal Diversity, FAO, Rome). Farmers were interviewed in detail to ascertain the unrelatedness of sampled individuals. Blood samples were collected by jugular venipuncture into EDTA coated vacutainers. Genomic DNA was extracted by standard phenol-chloroform method. A set of 27 microsatellite markers recommended by FAO (Table 1) and the International Society for Animal Genetics (FAO, 2011) for diversity analysis in cattle were utilized for genotyping. The forward primer for each locus was labelled with one of the three

fluorescent dyes FAM, HEX and ATTO550 (Applied Biosystems, USA).

Polymerase chain reaction was performed with a total reaction volume of 20 µl, using the following thermal conditions, 95°C for 15 min, followed by 40 cycles of 95°C for 50s, specific annealing temperature for 45s and 72°C for 45s and a final extension at 72°C for 10 min. The PCR composition include, 0.6 µl of forward and reverse primer each at a concentration of 5 pmol/µl, 2 µl each of dNTPs (2 mM) and 10× buffer II (Qiagen), 0.15 µl of Taq polymerase (Solis) at a concentration 5U/µl, 12.65 µl of molecular biology grade water and 2 µl of DNA. The amplified PCR products containing different dyes were then electrophoresed together after multiplexing in six sets (Table 1) in an automated DNA sequencer along with ROX500 (Applied Biosystems, USA) as an internal lane control. The allele size data for each sample was extracted using GENEMAPPER software.

Statistical Analysis: The basic diversity indices like observed number of alleles, allele frequency, observed and expected heterozygosity and inter-individual allele sharing distance were calculated using MICROSATELLITE ANALYZER (MSA) version 4.05 (Dieringer and Schotterer 2003). Wright's F-statistics including F_{IT} , F_{IS} and F_{ST} were calculated using FSTAT version 2.9.3. Deviations from Hardy-Weinberg equilibrium (HWE) were estimated by exact tests of heterozygote excess and deficit for each marker and population using GENEPOP software. Inter-individual allele sharing distance matrix was utilized to derive principal component that could describe the geometric relationship between cattle belonging to Alambadi and Bargur breeds. The first three largest principal components were plotted in a three-dimensional scatter diagram using SPSS version 13.0. Genetic bottleneck analysis was performed using BOTTLENECK program (Piry *et al.* 1999). Three tests, viz. sign test, standardized differences test and Wilcoxon signed rank test, were employed to test whether the population was deviating from mutation-drift equilibrium under different models of microsatellite evolution. A qualitative test for mode shift was also performed to detect whether the population has undergone any genetic bottleneck in the recent past.

RESULTS AND DISCUSSION

Genetic diversity and Hardy-Weinberg Equilibrium: A total of 176 and 188 alleles were observed among 27 microsatellite marker loci in Alambadi and Bargur cattle respectively. The mean observed number of alleles per locus in Alambadi was 6.52 and ranged from 3 (ETH3) to 11 (CSRM60) across different loci (Table 1). In Bargur cattle, the overall mean observed number of alleles per locus was slightly higher (6.96).

The genetic variability of Alambadi cattle in terms of mean observed and expected heterozygosity was 0.666 and 0.704 respectively, while it was 0.639 and 0.691 for Bargur cattle respectively (Table 2). The mean observed heterozygosity in Alambadi and Bargur cattle was

Table 1. Details of multiplex panels, PCR condition and allelic diversity of cattle microsatellite markers used in the study

Multiplex Panel	Locus	Dye	Annealing Temperature	Observed no. of alleles	
				Alambadi	Bargur
1	CSRM60	FAM	60°C	11	8
	CSSM66	FAM	60°C	9	8
	HEL1	HEX	56°C	6	6
2	INRA063	HEX	56°C	4	5
	BM1824	ATTO550	61°C	5	4
	ETH152	FAM	60°C	6	4
	HAUT27	HEX	54°C	7	5
	INRA05	FAM	54°C	5	6
3	BM1818	HEX	60°C	9	7
	ETH3	FAM	63°C	3	5
	HEL9	ATTO550	56°C	8	10
	ILSTS006	FAM	54°C	5	7
	TGLA53	HEX	55°C	9	8
4	HEL5	FAM	54°C	3	8
	HAUT24	HEX	53°C	8	7
	SPS115	FAM	61°C	6	6
	INRA032	ATTO550	56°C	9	8
5	ETH185	ATTO550	65°C	10	13
	ILSTS05	FAM	56°C	5	5
	INRA035	FAM	60°C	6	9
	HEL13	HEX	54°C	4	5
	TGLA126	HEX	54°C	7	7
6	BM2113	FAM	63°C	6	7
	ETH10	FAM	61°C	4	5
	ETH225	ATTO550	63°C	6	7
	INRA023	ATTO550	58°C	5	8
	TGLA122	HEX	58°C	10	10

comparable with Gir (0.679 ± 0.09), Deoni (0.674 ± 0.09) and Kankrej (0.674 ± 0.09 ; Kale *et al.* 2010). Even though Pulikulam cattle had a higher mean allelic diversity (7.89 ± 0.72) per locus, the observed heterozygosity (0.5758 ± 0.053 , Barani *et al.* 2015) was lower than Alambadi and Bargur cattle. Estimates of F_{IS} showed positive values in both Alambadi and Bargur cattle with a mean value of 0.056 and 0.076 respectively (Table 2). Significant heterozygosity deficit and potential inbreeding was observed in both the breeds with 18 out of 27 loci showing lower observed heterozygosity as expected out of Hardy-Weinberg proportions. However, the mean F_{IS} value observed in the present study was much lower than previous reports on several Indian cattle breeds like Kherigarh, Tharparkar, Gangatiri, Kenkatha and Pulikulam.

The test for Hardy-Weinberg equilibrium revealed 11.1% (3 out of 27) of the investigated loci showing significant deviations due to heterozygosity deficit and no locus showed deviation due to heterozygosity excess ($P < 0.05$). However, Bargur cattle showed higher proportion of loci deviating from HWE due to heterozygosity deficit (25.9%) (Table 3). Departure from HWE may result from one or more of the following reasons: (i) selective forces operating at certain loci, (ii) presence of null alleles, (iii) small sample size, (iv) Wahlund effect, i.e. presence of fewer

heterozygotes in a population than predicted on account of population sub division. Breeding of Alambadi and Bargur cattle are mostly done by natural service using native bulls available in the village or nearby areas. Artificial insemination is rarely practiced and availability of purebred semen from Alambadi and Bargur bulls is either absent or very limited. Hence, intense selection and breeding practices cannot be counted as potential factors for the observed heterozygosity deficit. However, it needs to be noted that the availability of quality purebred bulls (true to Alambadi and Bargur type) for natural breeding is very limited. Combined with the practice of castrating males to produce good quality bullocks, it is not uncommon that only one or few bulls being available for several breedable females across several villages in the breed tract. Hence, higher observed heterozygosity deficit might have resulted due to potential consanguineous mating arising out of the usage of very few sires for natural breeding.

Fixation index and genetic structure: Genetic diversity between and within breeds was studied using Global F-statistics. The global F_{IT} , F_{IS} and F_{ST} were estimated to be 0.095, 0.067 and 0.03 respectively. The F_{ST} values ranged from -0.005 (ILSTS0) to 0.095 (TGLA12) across different microsatellite loci. The global F_{ST} indicated only 3% of the total variation. Further, to visualize genetic differentiation

Table 2. Observed heterozygosity, expected heterozygosity and estimated F_{IS} in Alambadi and Bargur cattle (F_{IS} – Estimated heterozygosity deficit)

Locus	Observed heterozygosity		Expected heterozygosity		F_{IS}	
	Alambadi	Bargur	Alambadi	Bargur	Alambadi	Bargur
CSRM60	0.889	0.652	0.834	0.644	-0.067	-0.013
CSSM66	0.852	0.857	0.808	0.784	-0.056	-0.095
HEL1	0.750	0.723	0.797	0.748	0.060	0.033
INRA63	0.679	0.612	0.528	0.571	-0.292	-0.073
BM1824	0.643	0.633	0.618	0.596	-0.042	-0.062
ETH152	0.556	0.245	0.577	0.226	0.038	-0.086
HAUT27	0.385	0.438	0.696	0.650	0.452	0.329
INRA05	0.679	0.653	0.782	0.806	0.135	0.192
BM1818	0.786	0.604	0.859	0.715	0.087	0.156
ETH3	0.556	0.532	0.444	0.574	-0.256	0.073
HEL9	0.815	0.917	0.865	0.884	0.059	-0.037
ILSTS006	0.552	0.646	0.618	0.695	0.109	0.071
TGLA53	0.667	0.370	0.753	0.443	0.116	0.168
HAUT24	0.654	0.447	0.747	0.631	0.126	0.294
HEL5	0.208	0.167	0.382	0.714	0.460	0.769
INRA032	0.815	0.737	0.836	0.783	0.026	0.060
SPS115	0.759	0.658	0.717	0.730	-0.059	0.100
ETH185	0.857	0.854	0.869	0.890	0.014	0.041
HEL13	0.375	0.532	0.548	0.563	0.320	0.056
ILSTS05	0.714	0.792	0.771	0.726	0.075	-0.091
INRA035	0.704	0.740	0.813	0.771	0.136	0.041
TGLA126	0.692	0.813	0.800	0.770	0.137	-0.056
BM2113	0.667	0.771	0.706	0.798	0.057	0.034
ETH10	0.655	0.633	0.653	0.696	-0.004	0.092
ETH225	0.655	0.700	0.675	0.725	0.029	0.035
INRA023	0.586	0.714	0.551	0.730	-0.066	0.021
TGLA122	0.828	0.820	0.773	0.795	-0.073	-0.032
Overall	0.666	0.639	0.704	0.691	0.056	0.076

Table 3. Test for Hardy–Weinberg equilibrium at different microsatellite loci in Alambadi and Bargur cattle (H_e –Heterozygosity)

Locus	P-value for H_e Deficit		P-value for H_e Excess	
	Alambadi	Bargur	Alambadi	Bargur
CSRM60	0.897	0.148	0.136	0.853
CSSM66	0.717	0.970	0.303	0.028
HEL1	0.350	0.334	0.650	0.668
INRA63	0.973	0.742	0.066	0.292
BM1824	0.682	0.839	0.428	0.189
ETH152	0.473	1.000	0.606	0.465
HAUT27	0.001	0.011	0.999	0.989
INRA05	0.068	0.004	0.934	0.995
BM1818	0.222	0.027	0.775	0.972
ETH3	0.960	0.369	0.088	0.659
HEL9	0.252	0.677	0.757	0.319
ILSTS006	0.496	0.158	0.575	0.840
TGLA53	0.006	0.066	0.993	0.937
HAUT24	0.078	0.038	0.919	0.964
HEL5	0.006	0.000	0.999	1.000
INRA032	0.237	0.083	0.765	0.919
SPS115	0.127	0.010	0.875	0.990
ETH185	0.409	0.158	0.608	0.842
HEL13	0.059	0.231	0.957	0.807
ILSTS05	0.155	0.815	0.849	0.207
INRA035	0.155	0.022	0.850	0.981
TGLA126	0.262	0.772	0.744	0.243
BM2113	0.493	0.311	0.564	0.686
ETH10	0.490	0.197	0.513	0.811
ETH225	0.151	0.189	0.854	0.814
INRA023	0.859	0.587	0.193	0.411
TGLA122	0.911	0.465	0.160	0.530

among the two breeds, principal components analysis (PCA) was employed. A total of 22 principal components were extracted, each with eigen values greater than one and collectively explaining 83.7% of total variance in the dataset (Fig. 1.). The three largest principal components (PC1, PC2 and PC3) explained 10.9%, 10.4% and 8.02% respectively and cumulatively 29.32% of the total variation. The three-dimensional scatter diagram derived from these three largest principal components showed two distinct centroids respectively for Alambadi and Bargur cattle. However, overlapping of few individuals were observed indicating dispersal and genetic admixture among the two studied breeds (Fig. 2). This is understandable, as there is considerable geographical overlapping of the native tracts of both these cattle breeds, particularly their predominant grazing areas situated around the hilly and forest regions of Western Ghats bordering the states of Tamil Nadu and Karnataka.

Test for mutation drift equilibrium. The population of Alambadi, a breed being neglected due to its low milk production, showed a rapid decline in size over the last three decades. Hence, mutation drift equilibrium was assessed to identify any potential evidence for the recent occurrence of genetic bottleneck in the population. Three different mutation models of microsatellite evolution were assumed,

viz. infinite alleles model (IAM), stepwise mutation model (SMM) and two-phase model (TPM). Assumption of SMM and TPM revealed highest (0.762 ± 0.011) and lowest (0.689 ± 0.018) mean expected equilibrium gene diversity over 27 microsatellite markers in Alambadi cattle. Under TPM, the calculated mean equilibrium gene diversity was intermediate (0.728 ± 0.014). Since IAM always has a lower expected equilibrium gene diversity estimate as compared to more conservative SMM, this result is expected (Kataria *et al.* 2010).

Sign test, standardized differences test and Wilcoxon sign rank test were performed under each mutation model. Out of 27 loci, 16 loci were expected to show heterozygosity excess in Alambadi cattle. Under IAM, observed number of loci (16) showing heterozygosity excess was at par with expected number, but in SMM and TPM observed number of loci with heterozygosity excess was considerably lower (9 and 10 respectively) than expected. Sign test did not reveal significant heterozygosity excess ($P < 0.01$) across all the three mutation models, indicating Alambadi cattle was not deviating from mutation drift equilibrium (Table 4). Standardized difference test revealed positive T_2 value

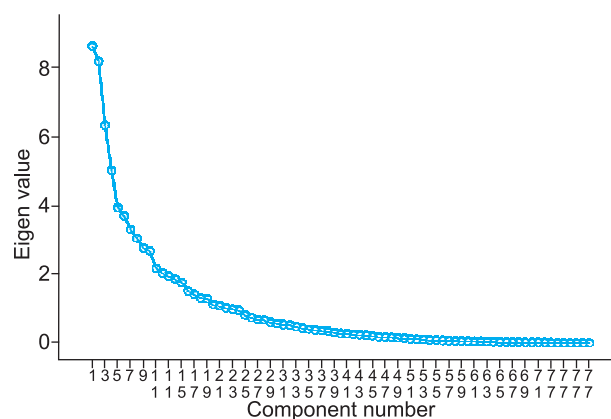


Fig.1. Scree plot showing proportion of variance explained by each principal component extracted from pairwise inter-individual allele sharing distance among Alambadi and Bargur cattle.

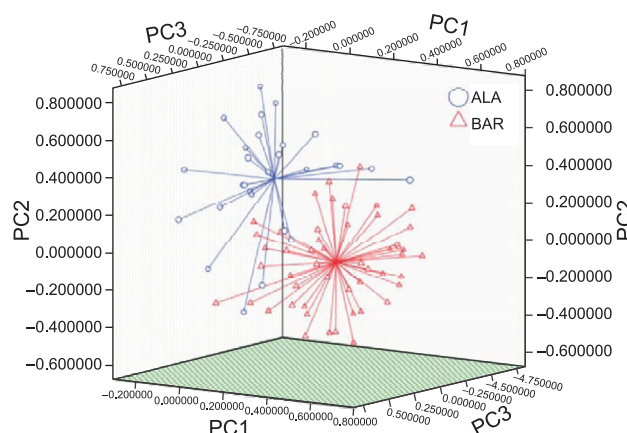


Fig. 2. Scattergram derived from three largest principal components extracted from pairwise inter-individual allele sharing distance among Alambadi and Bargur cattle.

Table 4. Tests for genetic bottleneck in Alambadi and Bargur cattle (IAM–Infinite Allele Model; SMM–Stepwise Mutation Model; TPM–Two Phased Mutation Model; H_e –Heterozygosity)

Breed	Test	Parameter	IAM	SMM	TPM
Bargur	Sign test	Expected no. of loci with H_e excess	16.06	16.00	16.10
		Observed no. of loci with H_e excess	21	6	13
		P-value	0.037	0.000	0.154
	Standardized differences test	T_2 value	1.60	–6.76	–1.56
		P-value	0.055	0.000	0.059
Alambadi	Wilcoxon signed rank test	Probability of one-tail test (H_e excess)	0.006	0.999	0.711
	Sign test	Expected no. of loci with H_e excess	16.11	16.15	16.10
		Observed no. of loci with H_e excess	16	9	10
		P-value	0.556	0.005	0.015
	Standardized differences test	T_2 value	1.26	–4.87	–1.24
		P-value	0.104	0.000	0.107
	Wilcoxon signed rank test	Probability of one-tail test (H_e excess)	0.064	0.999	0.832

under IAM (1.26) and negative values under TPM (–1.24) and SMM (–4.87). Similarly, Wilcoxon sign rank test revealed no significant deviation ($P < 0.05$) from mutation drift equilibrium in Alambadi cattle. In case of Bargur cattle, all the three statistical tests revealed significant heterozygosity excess and deviation from mutation drift equilibrium under IAM. However, none of these tests revealed significant deviations when assumed under SMM or TPM.

To further assess the genetic bottleneck in Alambadi and Bargur cattle, a qualitative test was carried out by plotting proportion of different alleles against allele frequency class. In the event of recent genetic bottleneck, the loss of rare alleles is expected to distort the normal L-shaped distribution of allele frequencies. In the present study, no such mode shift was observed in both Alambadi and Bargur cattle, further reiterating the absence of genetic bottleneck in the recent past (Fig.3.). The results observed in the present study on Bargur cattle was contradictory to Ganapathi *et al.* (2012), who reported significant deviations from mutation drift equilibrium across all the mutation models when tested with all the three statistical procedures. They also reported mode shift following the qualitative test on allele frequency distribution indicating potential loss of rare alleles in Bargur cattle. This is quite surprising considering the fact that the samplings for both the studies were conducted only at an interval of few years. However, genotyping methodologies differed across these two studies: Ganapathi *et al.* (2012) utilized manual denaturing PAGE and silver staining procedure while the present study utilized automated capillary electrophoresis for genotyping and allele calling. Several reports have indicated that the manual technique detected fewer alleles and produced less consistent results as compared to automated procedure and the latter is the preferred choice for microsatellite genotyping (Stewart *et al.* 2011; Ellis *et al.* 2011). Although the transient heterozygosity excess and the loss of rare alleles reported by Ganapathi *et al.* (2012) could be due to technical issues in genotyping, further studies with additional markers and expanded sample size are required

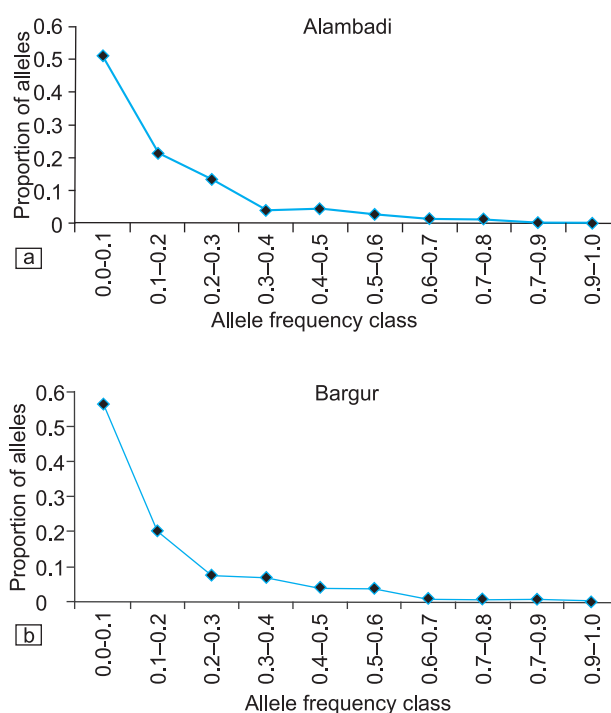


Fig. 3 (a-b). Qualitative test for genetic bottleneck based on allele frequency distribution in Alambadi and Bargur cattle.

to evaluate the possible occurrence of genetic bottleneck in Bargur cattle.

The reduced use of the breed in agricultural operations has had negative impact on farmers who are traditionally rearing these animals. Hence, the initiative of the Department of Animal Husbandry of the state of Tamil Nadu to establish a research and conservation centre for Alambadi cattle is timely. It is worth to mention that additional efforts are necessary to make the farmers aware of scientific breeding practices and limit the levels of inbreeding in such small populations. Availability of purebred semen from Alambadi cattle for artificial insemination will help farmers to maintain the breed purity and conserve this important draught cattle germplasm. From conservation standpoint,

further studies are required to understand taurine introgression in Alambadi cattle and its genetic structure/relationship with other draught cattle breeds of South India.

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