Breed traceability of buffalo meat using microsatellite genotyping technique

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Abstract Although buffalo has emerged as a major meat producing animal in Asia, major research on breed traceability has so far been focused on cattle (beef). This research gap on buffalo breed traceability has impelled development and validation of buffalo breed traceability using a set of eight microsatellite (STR) markers in seven Indian buffalo breeds (Bhadawari, Jaffa-arabadi, Murrah, Mehsana, Nagpuri, Pandharpuri and Surti). Probability of sharing same profile by two individuals at a specific locus was computed considering different STR numbers, allele pooling in breed and population. Match probabilities per breed were considered and six most polymorphic loci were genotyped. Out of eight microsatellite markers studied, markers CSSMO47, DRB3 and CSSM060 were found most polymorphic. Developed technique was validated with known and unknown, blood and meat samples; wherein, samples were genetically traced in 24 out of 25 samples tested. Results of this study showed potential applications of the methodology and encourage other researchers to address the problem of buffalo traceability so as to create a world-wide archive of

breed specific genotypes. This work is the first report of breed traceability of buffalo meat utilizing microsatellite genotyping technique.

Keywords Traceability · Buffalo meat · Breed · Microsatellite · Buffalo

Introduction

India is the highest beef exporter in the world. Due to restrictions on export of cattle meat, all the beef exported from India is from water buffalo (*Bubalus bubalis*). In 2015–2016, India exported 1.37 million tons of buffalo meat worth USD 4 billion (APEDA 2016). But most of the buffalo meat produced in India is from spent animals which have completed their productive and reproductive life. There are about 17 buffalo breeds in India registered under Genetic resource database of National Bureau of Animal Genetic Resources (NBAGR), Karnal, India. But there are no exclusive meat purpose buffalo breeds in India. As per Maurice et al. (2016) size of the water buffalo herd, including the female herd in India, is expected to begin shrinking in 2020s making growth in buffalo meat export unsustainable. To counter this trend there is a need to develop meat purpose breeds of buffaloes and undertake male buffalo calf rearing for meat production.

Traceability is a system of maintaining credibility of identification of animals or their products across the food supply chain i.e. farm-to-retailer (McKean 2001). Conventional traceability system based on paper documents could be counterfeited while genetic traceability is based on the identification of both animal and their products through the study of DNA and therefore is more reliable (Sardina et al. 2015). Unlike geographical traceability, genetic traceability

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identifies individual, breed or species (animal or its products) using DNA signatures. Since certain breed names have emerged as brand names in cattle, it has fueled interest among stakeholders of the meat industry to develop techniques for the certification of meat products including breed of origin (Narrod and Fuglie 2000). Nevertheless, mainstream research in breed traceability has been directed towards cattle meat (beef); while buffalo meat has largely been neglected in spite of its promising share in the Asian or Indian meat industry. As India is the highest exporter of buffalo meat in the world, developing breed traceability system for Indian buffalo breeds is extremely relevant owing to its export potential. Breed traceability of buffalo meat will help in identification of origin of breed thereby triggering the development of meat breeds suitable for diverse agro-climatic conditions. In addition, breed traceability can also help to ensure food safety, authenticity and preference of consumers for meat from specific breeds. Keeping these issues in mind, present study was planned to develop DNA based breed verification technique by microsatellite genotyping using a panel of microsatellite markers.

Materials and methods

Sample collection

Blood samples (~10 mL) were drawn from jugular vein into ethylene diamine tetra acetate (EDTA)—coated tubes from seven Indian buffalo breeds i.e. Bhadawari (n = 20), Jaffarabadi (n = 24), Murrah (n = 20), Mehsana (n = 15), Nagpuri (n = 15), Pandharpuri (n = 45) and Surti (n = 10) from their respective breeding tracts. For validation of the breed traceability study 15 blood samples from Pandharpuri breed and 10 blood samples of Murrah breed were collected from their native tract and 10 meat samples were collected from Municipal slaughter houses located at Hubli, Karnataka, India and Chengicherla, Hyderabad, India, respectively. Blood and meat samples were brought to laboratory under chilled conditions (0–5 °C) for DNA isolation.

DNA isolation

Genomic DNA was extracted from meat samples by phenol: chloroform method (Sambrook and Russel 2001); while commercial DNA extraction kit (Chromous Biotech, Bengaluru, India) was used for blood samples.

Microsatellite genotyping

Panel of microsatellite markers for buffalo (*Bubalus bubalis*) species was selected based on the PIC

(Polymorphism Information Content) score as recommended by the International Society for Animal Genetics (ISAG)/Food and Agricultural Organization (FAO) (2011). Selected microsatellite markers were custom synthesized (Chromous Biotech Bangalore) and 5' ends of forward primers was labeled with either FAM (6 carboxyfluorescein) or HEX (hexachloro-6-carboxyfluorescein) (Applied Biosystems, Foster City, CA) fluorescent dyes; while reverse primers were kept un-labeled. Table 1 shows sequence, chromosome location, annealing temperature, Genbank accession number and allele range of the selected primers.

PCR reactions were standardized for eight sets of microsatellite markers. Amplified PCR products were analyzed using horizontal submarine gel electrophoresis. Post-PCR multiplexing was performed simultaneously to genotype 2 loci, depending on the amplified PCR product size and primers were labeled with dye. Fluorescently end-labeled microsatellite PCR product-multiplex mix were then run on ABI Prism 3500xL capillary based genetic analyzer against Liz 500R (Applied Biosystems) as the internal lane standard (size standard). Raw data collected using 310 Data collection Software Version 3.1.0. Microsatellite fragment sizing was analyzed by Gene MapperTM version 3.7 (Applied Biosystems).

Statistical analysis

Polymorphism Information Content (Botstein et al. 1980), and genetic distances (Nei 1972) were calculated using Microsatellite Analyzer GenAlEx 6.5—Genetic Analysis in Excel software (Peakall and Smouse 2012). Markers selected for breed traceability were evaluated for match probability. The match probability (Weir 1996) is defined as the probability of two groups of animals (breed) sharing identical allelic profile by chance and was estimated as the product of genotype frequencies assuming that samples came from unrelated animal group. Individual identification by match probability formula was calculated according to Weir (1996).

Results and discussion

All 149 blood samples of unrelated seven buffalo breeds collected from breeding tracts of respective breeds were microsatellite genotyped using eight set of microsatellite markers. Polymorphism Information Content (PIC) and Match Probability (MP) are the two important factors which are considered for employing microsatellite markers for molecular traceability (Dalvit et al. 2008; Caliebe et al. 2015; Roewer et al. 2000).

Table 1 Microsatellite marker panel information

Primer		Primer sequence (5′–3′)	Label	Bases	Tm	Chromosome ^a	AT (°C)	Accession number	Allele range (bp)
1. CSSM033	F	cac tgt gaa tgc atg tgt gtg agc	FAM	24	64.63	17 (17)	65	U03805	154–175
	R	ccc atg ata aga gtg cag atg act	–	24	60.33				
2. CSSM047	F	tct ctg tct cta tca cta tat ggc	HEX	24	53.19	3q (8)	55	U03821	127–162
	R	ctg ggc acc tga aac tat cat cat	–	24	62.17				
3. ETH003	F	gaa cct gcc tct cct gca ttg g	FAM	22	66.04	3p (19)	65	Z22744	96–192
	R	act ctg cct gtg gcc aag tag g	–	22	63.57				
4. CSSM061	F	agg cca tat agg agg caa gct tac	HEX	24	61.76	Unknown	60	–	100–126
	R	ttc aga aga ggg cag aga ata cac	–	24	59.73				
5. DRB3	F	gag agt ttc act gtg cag	FAM	18	47.47	2p (23)	50–55	–	142–198
	R	cgc gaa ttc cca gag tga gtg aag tat ct	–	29	68.75				
6. CSSM019	F	ttg tca gca act tct tgt atc ttt	HEX	24	55.99	1q (1)	55	U03794	131–161
	R	tgt ttt aag cca ccc aat tat ttg	–	24	59.93				
7. CSSM041	F	aat ttc aaa gaa ccg tta cac agc	FAM	24	59.44	21 (22)	55	U03816	129–147
	R	aag gga ctt gca ggg act aaa aca	–	24	62.98				
8. HMH1R	F	ggc ttc aac tca ctg taa cac att	HEX	24	58.24	21 (22)	60	D10197	169–187
	R	ttc ttc aag tat cac etc tgt ggc c	–	25	63.51				

Tm melting temperature, *HEX* hexachloro-6-carboxyfluorescein, *FAM* 6-carboxyfluorescein

^a Cattle chromosome assignments in parentheses. *AT* annealing temperature

Polymorphic information content (PIC)

In the present study, PIC value observed in Bhadawari breed for markers CSSM047, CSSM033 and CSSM19 were 0.57, 0.51 and 0.67, respectively and the mean PIC value was 0.60 (Table 2) whereas, Tania et al. (2006) reported different PIC value for the same breed and for same marker were 0.80, 0.65, 0.47, respectively with a lower mean PIC value of 0.57. Moiola et al. (2001) studied genetic diversity of Italian and Greek buffalo populations and observed that, out of nine selected microsatellite markers CSSM047, DRB3 and CSSM060 were most polymorphic. The first two microsatellite markers (CSSM047 and DRB3) were most polymorphic in the present study also. Mean PIC value observed across the loci and breeds in the present study was in accordance with Dalvit et al. (2008). All eight loci studied were found to be

moderate to highly polymorphic. Out of the eight microsatellite markers studied, two markers viz., ETH003 and HMH1R showed overall PIC of 0.40 and 0.36 respectively. Due to low PIC these markers were not considered for further study while remaining six markers were used for studying match probability and breed authentication of buffalo meat. Markers showing PIC values higher than 0.5 are considered highly polymorphic (Botstein et al. 1980). Five of the six microsatellite markers selected for traceability studies showed overall PIC values of above 0.5 in the present study (Table 2).

Match probability

The match probability (MP) is defined as the probability of two groups of animals (breeds) sharing an identical allelic profile by chance and was estimated as the product of

Table 2 Polymorphism information content (PIC) values for microsatellite markers among different buffalo breeds

	Bhadawari	Jaffarabadi	Murrah	Mehsana	Nagpuri	Pandharpuri	Surti	Overall
1. CSSM033	0.51	0.45	0.47	0.22	0.37	0.70	0.49	0.45
2. CSSM047	0.57	0.82	0.77	0.70	0.79	0.81	0.78	0.74
3. ETH003	0.37	0.37	0.41	0.37	0.46	0.45	0.37	0.40
4. CSSM061	0.76	0.78	0.71	0.75	0.80	0.83	0.75	0.76
5. DRB3	0.80	0.71	0.75	0.37	0.79	0.86	0.46	0.67
6. CSSM019	0.67	0.72	0.83	0.63	0.81	0.76	0.60	0.71
7. CSSM041	0.74	0.62	0.62	0.49	0.64	0.82	0.42	0.62
8. HMH1R	0.41	0.37	0.35	0.36	0.37	0.48	0.22	0.36
Overall	0.60	0.60	0.61	0.48	0.62	0.71	0.59	

Table 3 Match probability of finding two identical individuals calculated using a different number of microsatellites (N)

N	Bhadawari		Jaffarabadi		Murrah		Mehsana		Nagpuri		Pandharpuri		Surti	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
2	2.8E-03	1.4E-02	2.1E-03	2.1E-03	2.4E-03	6.1E-03	7.4E-03	7.4E-03	2.5E-03	2.5E-03	1.1E-03	1.9E-03	5.7E-03	5.7E-03
3	2.2E-04	1.7E-03	2.0E-04	2.0E-04	1.9E-04	2.4E-04	1.1E-03	1.1E-03	1.4E-04	1.4E-04	4.6E-05	1.2E-04	7.2E-04	7.2E-04
4	2.7E-05	6.3E-05	1.9E-05	1.9E-05	1.9E-05	1.9E-05	2.8E-04	4.2E-04	7.9E-06	7.9E-06	2.2E-06	3.3E-06	1.9E-04	2.1E-04
5	5.0E-06	5.0E-06	3.0E-06	3.0E-06	2.8E-06	2.8E-06	1.0E-04	1.1E-04	1.1E-06	1.1E-06	1.4E-07	1.4E-07	5.6E-05	7.0E-05
6	1.2E-06	1.2E-06	8.7E-07	8.7E-07	7.8E-07	7.8E-07	4.1E-05	6.4E-05	3.2E-07	3.2E-07	1.5E-08	1.5E-08	1.8E-05	1.8E-05

Column A: markers are chosen according to their PIC values in the considered breed

Column B: markers are chosen according to their PIC values in the pooled population of breed

genotype frequencies assuming that samples come from unrelated animals group (Weir 1996). The values of Match Probability was calculated using 6 marker sets namely CSSM061, CSSM047, CSSM019, DRB3, CSSM041 and CSSM033; starting with most polymorphic marker and adding sequentially the next most polymorphic (Table 3). The match probability value obtained in Bhadawari, Jaffarabadi, Murrah, Mehsana, Nagpuri, Pandharpuri and Surti was in the range from a of 1.5×10^{-8} (0.015 individuals out of 1 million with same genotype) for Pandharpuri to maximum of 1.8×10^{-5} (18 individuals out of 1 million with same genotype) for Surti, whereas MP value observed when microsatellite observed in pooled animals was in the range from a minimum of 1.5×10^{-8} (0.015 individuals out of 1 million with same genotype) for Pandharpuri to maximum 6.4×10^{-5} (64 individuals out of 1 million with same genotype) for Mehsana. Match probability was good enough for utilization of the technique for breed traceability of buffalo meat.

Validation of developed technique with known blood samples

In order to validate the developed technology for breed authentication of buffalo meat, 25 blood samples were collected from known buffalo meat breeds of which 15 samples were Pandharapuri and ten samples were from Murrah breed of buffaloes. All the samples were microsatellite genotyped using the selected six microsatellite markers viz., CSSM061, CSSM047, CSSM019, DRB3, CSSM041 and CSSM033. Microsatellite genotype results were matched with the genotype result of different buffalo breeds using STRUCTURE software analysis (Table 4). Out of the 25 known samples breed could be accurately assigned to 24 blood samples. One sample of Pandharapuri breed of buffalo showed more matching score to Murrah 0.386 and revealed second highest score of 0.24 with Pandharapuri blood samples. This may be due to occasional cross mating happening in field. While remaining 24 samples gave convincing highest score with expected breed of origin which indicated applicability of the test for breed traceability of buffalo meat. However, accuracy of the test can be further enhanced by increasing the number of microsatellite markers used for genotyping. This work is the first report of breed traceability of buffalo meat utilizing microsatellite genotyping technique.

Validation of the developed technique in unknown market buffalo meat samples

Efficiency of developed technique for breed identification of buffalo meat was validated by comparing market buffalo

Table 4 Breed assignment of meat and blood samples by microsatellite genotyping (matching score for each individual as per STRUCTURE analysis)

Sample code	Bhadawari	Jaffarabadi	Murrah	Mehasana	Napuri	Pandharapuri	Surti
<i>Blood samples</i>							
1. P06	0.038	0.055	0.091	0.024	0.038	0.725	0.028
2. P07	0.069	0.065	0.194	0.037	0.078	0.506	0.051
3. P08	0.202	0.074	0.169	0.050	0.053	0.433	0.019
4. P09	0.051	0.198	0.386	0.055	0.051	0.240	0.019
5. P10	0.029	0.017	0.056	0.016	0.024	0.845	0.013
6. P21	0.165	0.025	0.026	0.019	0.022	0.727	0.016
7. P22	0.017	0.013	0.019	0.011	0.012	0.919	0.010
8. P23	0.032	0.012	0.024	0.011	0.019	0.890	0.012
9. P24	0.022	0.031	0.033	0.029	0.035	0.835	0.016
10. P25	0.404	0.025	0.083	0.036	0.018	0.405	0.029
11. P26	0.035	0.010	0.025	0.013	0.010	0.896	0.010
12. P27	0.228	0.013	0.017	0.011	0.016	0.698	0.017
13. P28	0.087	0.024	0.049	0.016	0.034	0.766	0.025
14. P29	0.052	0.012	0.024	0.014	0.015	0.857	0.027
15. P30	0.094	0.021	0.030	0.027	0.019	0.782	0.028
16. MR11	0.096	0.032	0.691	0.031	0.033	0.089	0.028
17. MR12	0.119	0.028	0.631	0.030	0.034	0.137	0.021
18. MR13	0.057	0.020	0.835	0.016	0.016	0.032	0.025
19. MR14	0.091	0.017	0.765	0.017	0.031	0.056	0.023
20. MR15	0.098	0.059	0.523	0.044	0.064	0.167	0.045
21. MR16	0.129	0.042	0.529	0.032	0.048	0.158	0.062
22. MR17	0.030	0.025	0.839	0.012	0.028	0.034	0.032
23. MR18	0.092	0.014	0.679	0.029	0.016	0.150	0.020
24. MR19	0.033	0.018	0.838	0.014	0.026	0.058	0.012
25. MR20	0.150	0.022	0.684	0.016	0.041	0.056	0.031
<i>Meat samples</i>							
26. U01	0.042	0.055	0.103	0.027	0.039	0.702	0.032
27. U02	0.043	0.056	0.101	0.028	0.038	0.702	0.032
28. U03	0.034	0.012	0.043	0.013	0.012	0.875	0.011
29. U04	0.034	0.012	0.044	0.013	0.012	0.873	0.011
30. U05	0.015	0.085	0.014	0.330	0.101	0.036	0.420
31. U06	0.015	0.086	0.014	0.319	0.100	0.036	0.431
32. U07	0.094	0.151	0.046	0.041	0.514	0.055	0.100
33. U08	0.092	0.150	0.046	0.040	0.516	0.054	0.102
34. U09	0.040	0.088	0.039	0.116	0.497	0.044	0.176
35. U10	0.041	0.088	0.040	0.119	0.491	0.045	0.176

P Pandharapuri, *MR* Murrah, *U* meat samples from abattoir (unconfirmed breed)

meat samples of unknown breed, with the available data and distance (similarity or divergence) from different breeds. Ten meat samples were collected from abattoirs by noting the probable breed based on physical feature of the slaughter buffalo. DNA was extracted from the meat samples and microsatellite genotyping of the DNA was undertaken using panel of six microsatellite markers. Matching score calculated with the known breed genotype result of different breed could assign the breed origin of buffalo meat. Four samples were assigned to Pandharapuri breed, two samples were assigned to Surti breed while four

more samples were assigned to Nagpuri breed of buffaloes. These results were as per the phenotypic feature of the slaughter buffaloes. Mateus and Russo-Almeida (2015) developed a technique for breed traceability of 9 Portuguese cattle breeds by genotyping of 30 microsatellite markers which could trace breed origin with success rate of 97%. Due to the costs associated with obtaining enough data collection for a genotypic marker, the interest to minimize the number of loci required for correctly classifying the animals in their population of origin is obvious (Mateus and Russo-Almeida 2015) hence attempt was made in this

study to achieve breed traceability using six sets of microsatellite markers. Breed traceability can be achieved by microsatellite genotyping (Sardina et al. 2015; Mateus and Russo-Almeida 2015) and by SNP analysis (Orrù et al. 2009; Fontanesi et al. 2016). The use of SNPs in combination with Bayesian statistics for the breed traceability of cattle was evaluated using a dataset comprising 24 breeds from Italy, France, Spain, Denmark, the Netherlands, Switzerland and UK genotyped with 90 polymorphic markers by Negrini et al. (2008) but the major problem in traceability using this method is high cost (Orrù et al. 2009). Moreover, SNP analysis will be highly useful only if the SNP database of the targeted species is available. As not much literature is available on SNP in buffalo breeds microsatellite genotyping based method will be of help for achieving breed traceability of buffalo meat.

Conclusion

The results showed that the statistical approach based on the match probability and genetic identity together with a choice of STR showing high gene diversity, enabled the achievement of the satisfactory assignment rate. Implementation of a reliable traceability system does not depend only on the choice of the most appropriate markers but is strongly influenced by the population one is tracing. Breeds characterized by weak genetic differentiation are more difficult to discriminate even using the most appropriate marker set. The results suggest that, when the aim is to distinguish between closely related breeds, future research should focus on the detection of diagnostic markers showing the presence of private alleles. Our results highlight the real potential of the methodology and encourage other researchers to address the problem so as to create a world-wide archive of individual genotypes.

In conclusion, market meat could be traced to its breed using multi loci genotyping of microsatellite markers. However, further studies are warranted to prove applicability of this technique for diverse buffalo breeds of the world.

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