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AFLP based Breed Marker Present a Decree for Pakistani Sahiwal Cattle Breed Identification

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Key words
Molecular markers, Breed identification, Crossbred

ABSTRACT

Molecular identification of animals is becoming increasingly important to preserve and maintain pure breeds worldwide. The issue is aggravated with rise in import of foreign animals and germplasm in Pakistan. It is becoming difficult to find pure males of Sahiwal breed for breeding purpose in public as well as private semen production units. The present study was designed to develop standard molecular markers for Sahiwal to ascertain their purity for breeding purpose. In this study 50 and 48 unrelated males were sampled for each Sahiwal and Crossbred cattle respectively. Candidate molecular markers present in Sahiwal but absent in Crossbred and vice versa were detected using amplified fragment length polymorphism method. Eleven markers were developed that were converted to single nucleotide polymorphism markers for high throughput genotyping. The allele frequencies in both breeds were determined for discrimination ability using AFLP. The probability of identifying Sahiwal breed was 0.86 and probability of misjudgment was 0.021 using single selected marker. However, probabilities for judgment and misjudgment with two markers and combined with three markers were 0.745, 0.367 and 0.964, 0.376 respectively. The results demonstrated that Sahiwal breed and crossbred could be tested using the given markers and can be verified for purity before entering into breeding program.

INTRODUCTION

Pakistani cattle breeds fall under group of zebu cattle (*Bos indicus*). These are categorized into dairy, draft and dual-purpose breeds depending upon their utility either in dairying or in agricultural work. The specific features credited to local breeds are characters i.e. disease resistance, heat tolerance, ability to survive and reproduce under stress and low input system. There are 35.6 million cattle in the country with a positive population growth rate. However, more than half of the cattle population does not belong to any specific breed group and thus categorized as non-descript. Moreover, Sahiwal, Red Sindhi and Cholistani are the distinguished dairy cattle breeds. Thari (also called as Tharparkar) is a dairy-cum draught breed. The draught breeds include Bhagnari, Dajal, Dhanni, Lohani, Rojhan and Kankaraj. Phenotypic characterization is available for most of the breeds and among dairy cattle breeds, Sahiwal is most studied breed compared to Red Sindhi, Cholistani, Tharparkar and other cattle breeds (Afzal and Naqvi, 2004). Most of these studies pertain to phenotypic and genetic parameters at population level. However, the accuracy of phenotypic characterization of domestic cattle is often affected by the influence of the environment and the underlying genetic complexity.

Genetic characterization at molecular level is very preliminary. There are few studies that focus mainly on genetic variation and diversity in cattle (Azam *et al*., 2012; Imran *et al*., 2012; Nasreen *et al*., 2012). Genetic diversity of Hariana and Hissar cattle breeds of Pakistan was investigated using 30 bovine microsatellite markers. It was concluded that although Hariana and Hissar breeds shared the common breeding tract, yet these are genetically different enough to be identified as two separate breeds

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(Rehman and Khan, 2009). In another study, diversity of Tharparkar and Red Sindhi was studied using microsatellite markers (Azam *et al*., 2012). The review revealed that no work has been done to find out breed specific markers in Pakistani dairy cattle breeds.

A number of studies have been initiated to characterize the European cattle breeds in 90s that continues to date using the molecular tools like microsatellite markers (Bradley *et al*., 1996; Kantanen *et al*., 2000; Canon *et al*., 2001; Ginja *et al*., 2010; Cooper *et al*., 2016). Moreover, within the last decade, significant progress in molecular technology has made it possible to perform genetic analysis based on DNA markers. In livestock animals, DNA markers have been used for pedigree registration, individual identification, parentage testing and removal of carrier individuals with genetic diseases.

Although, over the period of time; breed identification methodology has been updated from simple methods i.e. AFLP markers to genomic chip (Cooper *et al*., 2016; Gurgul *et al*., 2016) however, AFLP (amplified fragment length polymorphism) method is one of the cheapest way to provide these useful markers (Milanesi *et al*., 2008). Since many polymorphic bands can be detected using combinations of selective primers, AFLP is a powerful method for acquiring genome information easily. It has been widely applied for genetic relationship studies (Negrini *et al*., 2006), QTL analysis (Milanesi *et al*., 2008), linkage mapping (Huang *et al*., 2009) and profiling of gene expression using cDNA (Pareek *et al*., 2012). Sasazaki *et al*. (2007) reported the usefulness of AFLP markers as a tool to discriminate between domestic and imported beef.

Pakistani cattle breeds i.e. Sahiwal is very well adapted to the harsh climatic condition of the region and have golden characteristics of resistance against ticks and diseases. Furthermore, despite of the evolutionary significance of the Pakistani dairy cattle breeds, the available literature on identification of these breeds using reliable molecular markers is scanty. Molecular identification of cattle breeds is imperative to maintain pure breeds germplasm to avoid extinction by crossbreeding which is heavily and unrestricted practiced in the country at the moment. Furthermore, owing to uncontrolled wide scale crossbreeding, local precious genetic pool is at risk. It is becoming hard to find hundred percent pure males of local breeds for breeding purpose even on government farms. There has been no work done to find out breed specific markers in Pakistani dairy cattle breeds that can be used to distinguish between the breeds especially PCR based breed identification test is not available as its commonly available developed countries. Therefore, objective of the present research was to find breed specific standard molecular markers for genetic identification of dairy breeds viz. Sahiwal and crossbred to ascertain their purity for breeding purpose using PCR-AFLP.

MATERIALS AND METHODS

Animals and samples collection

In this study, 50 and 48 male animals from each of the two breed populations viz. Sahiwal and Crossbred were taken at random from different areas in the country following FAO guideline pertaining to selection and un-relatedness (FAO, 2011). Blood samples were collected in sterile tubes containing EDTA anticoagulant and samples were shipped to Molecular Genetics and Genomics Laboratory, PMAS-Arid Agriculture University Rawalpindi for further analyses.

DNA extraction

Genomic DNA was extracted from blood samples according to standard manufacture's protocols using GeneJET Whole Blood Genomic DNA Purification Kit (Thermo Scientific). The quality of DNA extracted was tested with Quawell 5000 Nanodrop and DNA was kept at -20°C until further used in the study.

AFLP method

The procedures of AFLP method were employed as described by (Vos *et al*., 1995). Sequence of AFLP adapters and primers are listed in Table I. Genomic DNA (500 ng) was digested with 5 U of *Taq I* (Invitrogen) at 65 ºC for 1 h, followed by second digestion with 5 U of *EcoR I* (Invitrogen) at 37 ºC for 1 h. Double-stranded adapters were ligated to the restriction fragments, following addition of 5 pmol *EcoR I* adapter, 50 pmol *Taq I* adapter, 1 mM ATP and 1 U of T4 DNA ligase at 37 ºC for 3 h. The ligated DNA fragment solution was then diluted 10-fold with 10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA and stored at -20 ºC.

Table I. Sequence of AFLP adapters and primers.

Name	Sequence (5'→3')
EcoRI adapter	CTC GTA GAC TGC GTA CC
	AAT TGG TAC GCA GTC TAC
TaqI adapter	GAC GAT GAG TCC TGA C
	CGG TCA GGA CTC AT
EcoRI primer + 1	GAC TGC GTA CCA ATT CA
TaqI primer + 1	GAT GAG TCC TGA CCG AC
	GAT GAG TCC TGA CCG AT
EcoRI primer + 3	GAC TGC GTA CCA ATT CAN N
TaqI primer + 3	GAT GAG TCC TGA CCG ACN N
	GAT GAG TCC TGA CCG ATN N

Pre-amplification was carried out in PCR machine where adapters were used as primer. This allowed a first selection of fragments by only amplifying the DNA restriction fragments that have been ligated to adapters on both ends. Pre-amplified fragments were preselected using 75ng each of *EcoR I* primer and *Taq I* primer with a single selective nucleotide and then reaction mixtures were diluted 10-fold with 10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA and stored at -20 ºC.

In order to restrict the level of polymorphism and to label the DNA, selective amplifications was performed using 5ng of EcoR I primer and 30 ng of Taq I primer with three selective nucleotides. PCR products amplified with different primer combinations were loaded onto 5.0% denaturing polyacrylamide gels and electrophoresed for 2 h and were detected by SilverXpress® Silver Staining Kit (ThermoFisher Scientific).

Afterwards, selected bands of selective amplicons were excised and purified using GeneJET Gel Extraction Kit (Thermo Scientific) using manufacturer's protocol. The extracted samples were used to carry out PCR under standard conditions with the primers used in the selective amplification of AFLP assays. The amplified PCR products were cloned by pUCM-T Cloning Vector Kit (Bio Basic Inc., Canada) according to the manufacturer's instructions and were transformed by heat shock method to DH5α and spread on a media in plate for overnight at 37 ºC. Positive colonies were picked up and cultured overnight in Luria–Bertani medium, and plasmids were isolated. Product size of the original DNA fragment was determined by restriction and electrophoresis. The plasmid was sequenced by Macrogen Korea.

Sequences analysis

All sequences were analyzed for homology to database using online site of the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) running the Blast programs (NCBI, 2002). Genotype information on sequenced fragments was obtained from blasting two breed sequences between them and across the whole genome with an average SNP spacing of 51.5 Kb with chromosome positions. Based on the genotyping data, allele frequency at each SNP was calculated and used to select candidate SNPs. Finally, the primers were designed on the SNP flanking site of selected SNPs. The region including the SNP was amplified using PCR methods. PCR were performed in a volume of 20 µL using DreamTaq Green PCR Master Mix (Thermo Scientific). PCRs were carried out using a standard PCR program with 2 min denaturation at 94°C, 30 cycles for 1 min at 94°C, 30 s annealing at temperatures, 30 s extension at 72°C, and final extension for 7 min at 72°C. The amplified fragments were converted to PCR-AFLP markers. The genotype frequencies of these makers on the subject animals were investigated in order to examine their applicability as breed specific markers.

Statistical analysis

In order to adequately evaluate the efficiency of these markers for discrimination between both dairy cattle breeds, the probability of identification was calculated based on the estimated allelic frequency of each marker. Analyses of Hardy Weinberg equilibrium (HWE) and likelihood ratio test of linkage disequilibrium, within breed diversity of haplotypes and expected heterozygosity were performed using the program Arlequin (Excoffier and Lischer, 2010). Moreover, probability of identification (*Pis*) and probability of misjudgment (*Pms*) of Sahiwal breed was calculated using different panels of markers i.e. using single marker, using two markers and using three markers. In case of two markers formula was $P_{is} = P_{ix} + P_{iy} - P_{ix} P_{iy}$ where *Pis* = Probability of identification of Sahiwal, *Pix* = Frequency of marker *x* in Sahiwal population and *Piy* = Frequency of marker *y* Sahiwal population and for probability of misjudgment was $P_{ims} = P_{ix} + P_{iy} - P_{ix} P_{iy}$ where *Pims* = Probability of misidentification, *Pix* = Frequency of marker *x* in Cross-bred population and *Piy* = Frequency of marker *y* Cross-bred population. Moreover, combined probability of all three markers was $P_i = P_{if} + (1- P_{if}) P_{is}$ *Pi* = Combined probability of identification of Sahiwal *Pif* = Probability of identification of single marker, *Pis* = Probability of identification of two whereas probability of misjudgment was calculated by $P_m = P_{mf} + (1- P_{mf}) P_{ms}$ where *Pm* = Combined probability of misidentification of Sahiwal, *Pmf* = Probability of misidentification with one marker and *Pms* = Probability of misidentification with two markers.

RESULTS AND DISCUSSION

Current study was designed to develop specific molecular markers that can differentiate between Sahiwal cattle and crossbred population. The AFLP approach followed was already described by (Sasazaki *et al*., 2006).

PCR-AFLP markers

Different molecular approaches have been used to identify breeds among different farm animal species over the period of time i.e. AFLP in cattle (Ajmone-Marsan *et al*., 1997; Sasazaki *et al*., 2004, 2006), microsatellite markers in dogs (Koskinen, 2003), microsatellite markers in goat (Iquebal *et al*., 2013), microsatellite markers in cattle (Rogberg-Munoz *et al*., 2014), allele-specific polymerase chain reaction in chicken (Choi *et al*., 2007) and SNP chip in cattle (Suekawa *et al*., 2010; Cooper *et*

al., 2014, 2016). AFLP markers has been widely applied in DNA finger printing (Vos *et al*., 1995; Ajmone-Marsan *et al*., 1997), genetic distance analysis (Ajmone-Marsan *et al*., 2002), QTL mapping (Milanesi *et al*., 2008), linkage mapping (Huang *et al*., 2009) and finally of course in breed identification (Sasazaki *et al*., 2004, Negrini *et al*., 2007a; b). However, in current study we adopted for AFLP markers as breed identification tool because these markers are more informative (Sasazaki *et al*., 2006). The detailed information on each of the marker used including their forward as well as reverse primers, annealing temperatures, product size and relevant mutation are given in Table II. Annealing temperature of all the eleven markers ranged between 59˚C and 65˚C. Product sizes of given markers ranged from 99bp to 570bp. The product sizes were different from the previously reported studies (Sasazaki *et al*., 2004, 2006) probably attributed to difference of breeds. Mutations corresponding to the given markers were the result of insertion/deletion of SNP. The size, location and corresponding gene information for each of the eleven marker is provided in Table III. Moreover, the chromosomal location of LABG3 and 4 remained unknown.

Within breed diversity of haplotypes and expected heterozygosity

AFLP markers can be used to estimate inbreeding as well as heterozygosity as reported earlier (Dasmahapatra *et al*., 2008). Within breed diversity of haplotypes and expected heterozygosity of each marker for Sahiwal and Crossbred cattle is given in Table IV. Gene diversity value was almost similar for Sahiwal and crossbred cattle (0.982±0.0004 vs. 0.981±0.0004). Expected heterozygosity for LABG4 was higher in Sahiwal than crossbred (0.494 vs. 0.396) but reverse was true in case of LABG8 (0.500 vs. 0.366) and LABG10 (0.430 vs. 0.077). Moreover, in Sahiwal only five markers showed heterozygosity whereas in Crossbred population 10 markers showed heterozygosity.

Genotype and allele frequencies

Genotype and allele frequencies were estimated using AFLP-PCR as given in Table V. The frequency of allele 1 ranged from 0.14 to 1.00 in Sahiwal population while 0.083 to 1.00 in case of crossbred population whereas, frequency of allele 2 ranged from 0.00 to 0.86 for Sahiwal and from 0.00 to 0.971 for crossbreds. The absence of PCR band indicated allele 1 whereas appearance of PCR and

Table II. Marker information for PCR-RFLP.

Marker	Neucleotide sequence (5'→3')	Annealing temperature (°C)	Product size (bp)	Mutations
LABG1	F: GAGTGTAGTTGATTTATTTTTATTTGT R: GAGTACTGACGCAGCACACCTACAGCC	65	170	6 bp insertion/deletion
LABG2	F: GTAAAACAACTTAGTGGTGAATTCGGG R: TCGGATTGCTTACGTGCCTTTCTGGAGAC	65	238	SNP at Ecor I site A → G
LABG3	F: CCTTTGTCTTCCACTGCCCACCTGTCA R: CACATCTCTTTAGCACTCTCGTTCTGGT	65	155	SNP at Taq I site G → A
LABG4	F: TAGGGAAGATACCACAATAAGTAAAG R: GTAAAGATAAACATGTAAAGATATAGCACAGCATCGACC	65	134	SNP at Taq I site A → G
LABG5	F: TGTTACAACGCAAGGCTGGGAAACTG R: GAGAGTGGAGAGAATAGCGGATGCCTCGACCTGACTTTC	65	190	SNP at Taq I site G → T
LABG6	F: CGGGCTGGTCTGAGAAAAGTCAAGTCAC R: CAGTCAATGAAGAGCCGAGTAGAAGAAC	65	570	1 bp insertion/deletion
LABG7	F: TCTTGGTCACCTGCTGCTTCCTGTCCTG R: CGTATCCGTAGTATAGTAGTATGGTG	63	498	SNP at Taq I site T → C
LABG8	F: ATTCTATCAACAGCAAAAACCAAGCATT R: AAATGGCAGGAAGGAAGGCTATAGATGG	63	99	1 bp insertion/deletion
LABG9	F: CCAAGGTCTAAGAGCCAGGGTACTGATGC R: TCTGTAAAGACAAAGTGAATCTCTAAGG	59	127	8 bp insertion/deletion
LABG10	F: ACCCCCGTCCTTCTTCCCCATCACAGCC R: GCAGACAACAGGAAGACCCGTAAGTTTC	65	99	3 bp insertion/deletion
LABG11	F: CACATGATACAGCAAAAGGAGTTC R: CCCAATGTTCTGACGTCTTCCGA	65	107	SNP T → G

Table III. The result of cattle genome BLAST (all assemblies Annotation Release 105) on each marker.

Marker	Size (bp)	Score	E value	Location	Gene
LABG1	170	121	4e-10	BTA-1	Thymosin beta-4
LABG2	238	231	2e-108	BTA-14	RNA-binding Raly-like protein
LABG3	155	121	2e-10	Un	ND*
LABG4	134	131	9e-59	BTA-5	Ras-related and estrogen-regulated growth inhibitor
LABG5	190	306	1e-71	BTA-5	BAG family molecular chaperone regulator 1
LABG6	570	991	0.0	BTA-11	Spermatid perinuclear RNA-binding protein
LABG7	498	714	0.0	Un	Similar to PTK2 protein
LABG8	99	66	1e-06	BTA-23	Hereditary hemochromatosis protein precursor
LABG9	127	70	2e-07	BTA-1	Golgin subfamily B member 1 isoform X1
LABG10	99	48	0.11	BTA-3	Chromodomain-helicase-DNA-binding protein 1-like
LABG11	107	113	2e-22	BTA-6	E3 ubiquitin-protein ligase LNX

* No corresponding gene found during genome blast

Table IV. Within breed diversity of haplotypes and expected heterozygosity of Sahiwal and cross-bred.

Breed	Diversity parameters		Expected heterozygosity	
Sahiwal	Sum of square freqs.	0.0200	Locus	Expected heterozygosity*
	Gene diversity	0.982±0.0004	LABG2	0.488
	Theta (Hom)	52.044±1.083	LABG4	0.494
	Theta (k)	13.186	LABG8	0.366
	Theta (S)	0.726±0.346	LABG10	0.077
	Theta (Pi)	1.665±1.084	LABG11	0.241
Cross-bred	Sum of square freqs.	0.0208	LABG2	0.219
			LABG3	0.187
			LABG4	0.396
	Gene diversity	0.981±0.0004	LABG5	0.458
			LABG6	0.117
	Theta (Hom)	49.85±0.06	LABG7	0.153
	Theta (k)	12.651	LABG8	0.50
	Theta (S)	1.461±0.528	LABG9	0.249
	Theta (Pi)	2.752±1.615	LABG10	0.43
			LABG11	0.04

* Results are only shown for polymorphic loci

indicated allele 2 in the current study. Therefore, allele 2 of LABG2, 4, 8, 10 and 11 are indicated as probable breed identification markers for Sahiwal. Similarly, allele 2 of LABG2-11 markers appeared to be crossbred specific markers. Similar methodology was previously adopted to identify Japanese black cattle and crossbred populations (Sasazaki *et al*., 2004, 2006).

Power of identification (Pis) *and misjudgment* (Pms)

Hardy-Weinberg equilibrium (HWE) was tested for each locus using genotypic results of the Sahiwal and Crossbred populations.. None of the loci showed significant departure from HWE at $P < 0.05$ for the probability test in the population. Linkage disequilibrium between a pair of loci was subsequently tested using a likelihood ratio test. No locus pairs showed significant disequilibrium at $P < 0.05$ (Tables VII and VII). Therefore, calculations of identification and misjudgment probability described below were based on assumption of no linkage among eleven markers (Sasazaki *et al*., 2006).

Table V. Allelic frequencies of Sahiwal and Cross-bred for 11 markers.

	Allelic frequency			
	Sahiwal		Cross-bred	
Locus	Allele1	Allele2	Allele1	Allele2
LABG1	1.00±0.00	0.00	1.00±0.00	0.00
LABG2	0.58±0.021	0.42±0.021	0.875±0.014	0.125±0.014
LABG3	1.00±0 .000	0.00	0.896±0.013	0.104±0.013
LABG4	0.44±0.021	0.56±0.021	0.729±0.019	0.271±0.019
LABG5	1.00±0.000	0.00	0.646±0.021	0.354±0.021
LABG6	1.00±0.000	0.00	0.937±0.011	0.063±0.011
LABG7	1.00±0.000	0.00	0.083±0.012	0.917±0.012
LABG8	0.76±0.018	0.24±0.018	0.521±0.022	0.0479±0.022
LABG9	1.00±0.00	0.00	0.854±0.015	0.146±0.015
LABG10	0.96±0.008	0.04±0.008	0.688±0.02	0.312±0.02
LABG11	0.14±0.015	0.86±0.015	0.979±0.006	0.021±0.006

Table VI. Standardized disequilibrium values (r^2) disequilibrium for Sahiwal population for two locus haplotypes.

Loci	r^2			χ^2 p value		
	Allele	1	2	Allele	1	2
Loci 1 and 3	1	0.051	0.051	1	0.00	0.00
	2	0.051	0.051	2	0.00	0.00
Loci 1 and 7	1	0.01	0.01	1	0.021	0.021
	2	0.01	0.01	2	0.021	0.021
Loci 3 and 7	1	0.015	0.015	1	0.005	0.005
	2	0.015	0.015	2	0.005	0.005
Loci 1 and 9	1	0.03	0.03	1	0.00	0.00
	2	0.03	0.03	2	0.00	0.00
Loci 3 and 9	1	0.001	0.001	1	0.563	0.563
	2	0.001	0.001	2	0.563	0.563
Loci 7 and 9	1	0.013	0.013	1	0.007	0.007
	2	0.013	0.013	2	0.007	0.007
Loci 1 and 10	1	0.015	0.015	1	0.004	0.004
	2	0.015	0.015	2	0.004	0.004
Loci 3 and 10	1	0.207	0.207	1	0.00	0.00
	2	0.207	0.207	2	0.00	0.00
Loci 7 and 10	1	0.051	0.051	1	0.00	0.00
	2	0.051	0.051	2	0.00	0.00
Loci 9 and 10	1	0.045	0.045	1	0.00	0.00
	2	0.045	0.045	2	0.00	0.00

Table VII. Standardized disequilibrium values (r^2) disequilibrium for cross-bred population for two locus haplotypes.

Loci	Allele	r^2		Allele	χ^2 value	
		1	2		1	2
Loci 1 and 2	1	0.017	0.017	1	0.003	0.003
	2	0.017	0.017	2	0.003	0.003
Loci 1 and 3	1	0.008	0.008	1	0.042	0.042
	2	0.008	0.008	2	0.042	0.042
Loci 2 and 3	1	0.003	0.003	1	0.212	0.212
	2	0.003	0.003	2	0.212	0.212
Loci 1 and 4	1	0.061	0.061	1	0.00	0.00
	2	0.061	0.061	2	0.00	0.00
Loci 2 and 4	1	0.101	0.101	1	0.00	0.00
	2	0.101	0.101	2	0.00	0.00
Loci 3 and 4	1	0.186	0.186	1	0.00	0.00
	2	0.186	0.186	2	0.00	0.00
Loci 1 and 5	1	0.027	0.027	1	0.0002	0.0002
	2	0.027	0.027	2	0.0002	0.0002
Loci 2 and 5	1	0.008	0.008	1	0.043	0.043
	2	0.008	0.008	2	0.043	0.043
Loci 3 and 5	1	0.001	0.001	1	0.404	0.404
	2	0.001	0.001	2	0.404	0.404
Loci 4 and 5	1	0.0001	0.0001	1	0.796	0.796
	2	0.0001	0.0001	2	0.796	0.796
Loci 1 and 6	1	0.013	0.013	1	0.009	0.009
	2	0.013	0.013	2	0.009	0.009
Loci 2 and 6	1	0.011	0.011	1	0.018	0.018
	2	0.011	0.011	2	0.018	0.018
Loci 3 and 6	1	0.034	0.034	1	0.00	0.00
	2	0.034	0.034	2	0.00	0.00
Loci 4 and 6	1	0.05	0.05	1	0.00	0.00
	2	0.05	0.05	2	0.00	0.00
Loci 5 and 6	1	0.006	0.006	1	0.074	0.074
	2	0.006	0.006	2	0.074	0.074
Loci 1 and 7	1	0.072	0.072	1	0.00	0.00
	2	0.072	0.072	2	0.00	0.00
Loci 2 and 7	1	0.036	0.036	1	0.00	0.00
	2	0.036	0.036	2	0.00	0.00
Loci 3 and 7	1	0.068	0.068	1	0.00	0.00
	2	0.068	0.068	2	0.00	0.00
Loci 4 and 7	1	0.006	0.006	1	0.087	0.087
	2	0.006	0.006	2	0.087	0.087
Loci 5 and 7	1	0.009	0.009	1	0.026	0.026
	2	0.009	0.009	2	0.026	0.026
Loci 6 and 7	1	0.084	0.084	1	0.00	0.00
	2	0.084	0.084	2	0.00	0.00

Table continue on next pages.....

Loci		r^2			χ^2 value	
	Allele	**1**	**2**	**Allele**	**1**	**2**
Loci 1 and 8	1	0.0005	0.0005	1	0.608	0.608
	2	0.0005	0.0005	2	0.608	0.608
Loci 2 and 8	1	0.02	0.02	1	0.001	0.001
	2	0.02	0.02	2	0.001	0.001
Loci 3 and 8	1	0.014	0.014	1	0.006	0.006
	2	0.014	0.014	2	0.006	0.006
Loci 4 and 8	1	0.035	0.035	1	0.00	0.00
	2	0.035	0.035	2	0.00	0.00
Loci 5 and 8	1	0.019	0.019	1	0.002	0.002
	2	0.019	0.019	2	0.002	0.002
Loci 6 and 8	1	0.016	0.016	1	0.004	0.004
	2	0.016	0.016	2	0.004	0.004
Loci 7 and 8	1	0.002	0.002	1	0.336	0.336
	2	0.002	0.002	2	0.336	0.336
Loci 1 and 9	1	0.0003	0.0003	1	0.696	0.696
	2	0.0003	0.0003	2	0.696	0.696
Loci 2 and 9	1	0.053	0.053	1	0.00	0.00
	2	0.053	0.053	2	0.00	0.00
Loci 3 and 9	1	0.00	0.00	1	0.885	0.885
	2	0.00	0.00	2	0.885	0.885
Loci 4 and 9	1	0.047	0.047	1	0.00	0.00
	2	0.047	0.047	2	0.00	0.00
Loci 5 and 9	1	0.147	0.147	1	0.00	0.00
	2	0.147	0.147	2	0.00	0.00
Loci 6 and 9	1	0.081	0.081	1	0.00	0.00
	2	0.081		2	0.00	0.00
Loci 7 and 9	1	0.064	0.064	1	0.00	0.00
	2	0.064	0.064	2	0.00	0.00
Loci 8 and 9	1	0.128	0.128	1	0.00	0.00
	2	0.128	0.128	2	0.00	0.00
Loci 1 and 10	1	0.003	0.003	1	0.205	0.205
	2	0.003	0.003	2	0.205	0.205
Loci 2 and 10	1	0.183	0.183	1	0.00	0.00
	2	0.183	0.183	2	0.00	0.00
Loci 3 and 10	1	0.008	0.008	1	0.041	0.041
	2	0.008	0.008	2	0.041	0.041
Loci 4 and 10	1	0.012	0.012	1	0.013	0.013
	2	0.012	0.012	2	0.013	0.013
Loci 5 and 10	1	0.001	0.001	1	0.387	0.387
	2	0.001	0.001	2	0.387	0.387
Loci 6 and 10	1	0.002	0.002	1	0.312	0.312
	2	0.002	0.002	2	0.312	0.312
Loci 7 and 10	1	0.02	0.02	1	0.001	0.001
	2	0.02	0.02	2	0.001	0.001
Loci 8 and 10	1	0.004	0.004	1	0.166	0.166
	2	0.004	0.004	2	0.166	0.166
Loci 9 and 10	1	0.01	0.01	1	0.024	0.024
	2	0.01	0.01	2	0.024	0.024

Allele 2 was identified to use as breed identification marker using PCR technique to discriminate between Sahiwal and crossbred populations in the country. The probability of judgment and misjudgment was calculated based on the frequency of marker LABG2, 4 and 8 in Sahiwal and crossbred populations. These three markers showed higher frequency in Sahiwal population whereas lower in crossbred population (Table VI). LABG2 showed probability of judgment of 0.860 and that of misjudgment of 0.021. However, combined probability of judgment for LABG2 and 4 was lesser to this (0.745) with higher degree of misjudgment (0.362). The probability of judgment of Sahiwal was improved using all three markers (0.964) however; this also raised the misjudgment as well (0.376). A single marker was strong enough to identify Sahiwal compared to previous study of as compared to previous study of Japanese black and crossbred cattle identification (Sasazaki *et al*., 2004, 2006).

Table VIII. Identification (*Pis*) and misjudgment (*Pms*) probabilities of Sahiwal breed using different panels of markers.

Markers panel	Identification probability (Pis)	Misjudgment probability (Pms)
LABG8	0.86	0.021
LABG2	0.42	0.125
LABG4	0.56	0.271
LABG2 + 4	0.745	0.362
LABG2+4+8	0.964	0.376

CONCLUSIONS

This research generated molecular breed specific markers to identify the purity of breeds under investigation. A facility for identification of Sahiwal cattle purity is established in Dept. of Animal Breeding and Genetics at PMAS-Arid Agriculture University, Rawalpindi to continue the extension work for farmers and other stakeholders for pure animals identification. Moreover, Sahiwal breeds could be tested genetically and verified for purity before entering into breeding program. Pure animals identification facility is open for farmers, government agencies i.e. LandDD, Livestock breed improvement departments and Livestock research farms to carry out breeding programs.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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