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THE USE OF AFLP-PCR MARKERS TO DISCRIMINATE BETWEEN RED SINDHI AND CROSSBRED DAIRY CATTLE OF PAKISTAN

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ABSTRACT

Breeds can be easily identified using molecular tools based on genomic architecture of breed within a species. Moreover, in developing countries like Pakistan where the danger of genetic erosion is a serious threat due to uncontrolled crossbreeding, the application of such tools is promising for breed identification and conservation. Thus, the current research was planned to prepare AFLP based genetic markers for distinction of Red Sindhi cattle from crossbred population. In current study, 50 and 48 unrelated (males as well as females) were sampled for Red Sindhi and crossbred cattle populations, respectively. AFLP method was used to fetch prospective molecular markers present only in Red Sindhi however, lacking in crossbred and vice versa. The analysis generated eleven markers that were translated to single nucleotide polymorphism markers for genotyping. These markers were used for allelic variation analysis in both sampled populations. Moreover, single selected markers generated an identification probability of 0.800 while a probability for misjudgment of 0.313 for Red Sindhi cattle. However, the combination of three markers yielded a probability for judgment of 0.941 while a probability of misjudgment was 0.413. The study yielded panel of AFLP markers for identification of Red Sindhi that can be used as breed purity test to discriminate between Red Sindhi and crossbred population.

Key words: AFLP markers, breed identification, Red Sindhi, Crossbred, Pakistan

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INTRODUCTION

The population of Zebu cattle (*Bos indicus*) in Pakistan is important like the majority of the developing countries cattle populations. Pakistani breeds are characterized as draft, dual-purpose and dairy breeds according to their utility as draught, dairy animal or both purposes. The explicit characters attributed to the local breeds are benefits i.e., disease, parasitic and heat resistance along with survival and reproduction under minimum feed availability scenario (low input system). The cattle population in Pakistan is 49.6 million heads and has increasing trend in term of population (GoP, 2020). However, 44% of cattle population is non-descript i.e., don't belong to any documented breed (Khan *et al.* 2008). Sahiwal, Cholistani and Red Sindhi are well recognized dairy breeds in Pakistan. Thari is dual-purpose breed and Bhagnari, Dajal, Dhanni, Lohani, Rojhan and Kankaraj are recognized as draught breeds. Genetics and environment do affect the precision of phenotypic characterization however, phenotypic characterization for all aforementioned breeds is available while Sahiwal is more scientifically explored breed in the country (Afzal and Naqvi, 2004).

The genetic study for breed characterization and identifications using AFLP was first reported in Cholistani cattle (Malik *et al.* 2018). However, there are handsome number of reports that primarily focused on variation because of genetic and diversity analysis among different cattle breeds from single locus to genome wide studies (Azam *et al.* 2012, Imran *et al.* 2012, Munir *et al.* 2017, Hussain *et al.* 2018, Mustafa *et al.* 2018, Peters *et al.* 2018, Iqbal *et al.* 2019). These studies reported microsatellite markers application to investigate the genetic diversity of Hariana and Hissar breeds of local cattle present in Pakistan indicating these two breeds as distinct breeds despite of common home tract (Rehman and Khan, 2009); diversity of Tharparkar and Red Sindhi breeds using microsatellite markers (Azam *et al.* 2012); genetic variation of interferon gema (Maryam *et al.* 2012); Pit gene variation in Sahiwal cattle (Munir *et al.* 2017); genome wide study for signature of selection (Mustafa *et al.* 2018) and genomic variation using whole genome analysis (Iqbal *et al.* 2019). The review disclosed that efforts has been made for molecular analysis of Pakistani breeds however, markers that could discriminate Pakistani dairy cattle breeds are yet to reported for Red Sindhi cattle.

It has been reviewed that molecular characterization of European cattle breeds was started during 90s and continued till recent 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). Thus, during last fifteen years and so, molecular genetics technology is revolutionized enough to generate DNA based testing markers for livestock species. Therefore, the developed markers are used to rectify issues related to the pedigree i.e., parentage identification, animal identification and culling of carrier animals for defected alleles.

Although, breed identification methodology has been shifted from simple method i.e. AFLP markers to genomic chip over the period of time (Cooper *et al.* 2016, Gurgul *et al.* 2016) however, AFLP (amplified fragment length polymorphism) method is still among the cheapest methods to develop genetic markers especially in developing countries (Milanesi *et al.* 2008). The main advantage of AFLP is the ability to generate highly informative polymorphic marker bands with the help of primers selection methodology that can unleash genomic information easily. AFLP has been applied to calculate genetic relations (Negrini *et al.* 2006), analyzing QTL (Milanesi *et al.* 2008), mapping linkages (Huang *et al.* 2009) and expression of genes using cDNA (Pareek *et al.* 2012). Sasazaki *et al.* (2007) testified the efficacy of AFLP markers for differentiation between local and imported beef cattle in Japan.

Pakistani Red Sindhi breed of cattle is comfortable with the severe weather conditions of the tropics and sub-tropics and has wonderful features of resistance against ticks and diseases. However, despite of the evolutionary and adaptive importance of the Pakistani Red Sindhi dairy cattle breed, the availability of information on molecular identification of this breed is missing. In light of available literature and the danger of cross breeding, it is quite pertinent to develop molecular identification markers for Red Sindhi cattle to respond the alarming situation when it will become difficult to locate pure bred males of Red Sindhi cattle for breeding purpose in the country. Thus, primary objective of the present research was to find breed specific molecular markers for genetic identification of Red Sindhi and crossbred animals to establish pureness of Red Sindhi for breeding purpose with the application of PCR-AFLP technique.

MATERIALS AND METHODS

Animals and sampling: Red Sindhi (n = 50) and Crossbred (a cross between Holstein Friesian and Red Sindhi; n = 48) animals were sampled respectively from both sexes at random from Government Livestock Farms located in Punjab and Sindh province of the country following guidelines of Food and Agriculture Organization (FAO) pertaining to selection and un-relatedness (FAO, 2011) in current study. These farms were Red Sindhi Cattle Breeding Farm, Tando Muhammad Khan, LES 205 TDA, Punjab and LES Qadirabad while sampling was carried out in May-June 2016. Ten mL blood sample was collected from jugular vein in sterile tube with coating of EDTA as anticoagulant from every animal. The collected samples were transported to Laboratories of Department of Animal Breeding and Genetics, PMAS-Arid Agriculture University Rawalpindi within 24 hours of collection in iceboxes.

DNA Extraction: Extraction of genomic DNA from whole blood samples was carried out according to typical manufacture's protocols using GeneJET Whole Blood Genomic DNA Purification Kit (Thermo Scientific). The extracted DNA was tested for quality and quantity using Quawell 5000 Nanodrop and DNA was stored at -80°C until further down steam processing and subsequent analysis.

AFLP methodology: The procedure of AFLP method was used as described by Vos *et al.* (1995). Sequences of AFLP primers along with adapters used are mentioned in Table 1.

Table 1: Sequence of AFLP adapters and primers reported by (Vos *et al.* 1995; Malik *et al.* 2018) in Pakistani cattle.

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

Taq I restriction enzyme (Invitrogen) was used for first digestion of genomic DNA as per manufacturer's protocol (at 65ºC for one hour) whereas second digestion was carried out using *EcoR I* restriction enzyme (Invitrogen) as per manufacturer's protocol (at 37ºC for one hour). The resulted fragments were ligated with adapter (double stranded) using T4 DNA ligase (Invitrogen) as per manufacturer's protocol and finally ligates were diluted tenfold in TAE buffer and kept at -20ºC till further processing.

Pre-amplification was carried out in PCR machine (Proflex PCR system from Applied Biosystems) by using adapters as primer to prime the ligated fragments generated from previous step. This made a first round of selection of restricted DNA fragments ligated at

both ends with adapters. Pre-amplified fragments were preselected using 75ng of product using *EcoR I* and *Taq I* primers with one selective nucleotide. The subsequent PCR products were diluted tenfold with TAE buffer and stored at -20 ºC until further processing. In next step, a selective amplification was performed using 5ng of each *EcoR I* and *Taq I* primers with nucleotides for selection of 3 nucleotides to confine the level of polymorphism and to label the DNA. These PCR products (amplicons) obtained using different sets of primers were run on denaturing poly acrylamide gels (5%) for two hours and bands were visualized by SilverXpress® Silver Staining Kit (Thermo Fisher Scientific). Finally, the specific bands produced as a result of previous selective amplification step were cut and cleaned by using GeneJET Gel Extraction Kit (Thermo Scientific) according to manufacturer's protocol. PCR was performed on these fragments purified in previous step and the amplicons were inserted into pUCM-T cloning vector (Bio Basic Inc., Canada). The prospective clones were transformed to DH5α by heat shock method cultured in plates overnight at 37 ºC to identify the positive clones. The plasmids from positive clones were extracted after culturing in Luria–Bertani medium for sixteen hours. Restriction and gel electrophoresis were carried out to identify the size of product before sending the plasmid for sequencing from Macrogen Korea.

Sequencing and analysis: The homology of sequencing products was carried out with online NCBI Blast tools freely available on http://www.ncbi.nlm.nih.gov/BLAST/ (NCBI, 2002). Genotype information for sequences were fetched by blasting both breeds sequences between themselves and across available whole genome sequence on NCBI with an average SNP spacing of 51.5 Kb along with chromosomal positioning of these fragments. The genotypic data yielded allelic frequencies on each SNP locus that were used to pick up prospective SNPs. Lastly; we designed the primer pairs against these SNPs on flanking regions SNPs. These regions containing SNPs were amplified in PCR machine. PCR was carried out with a volume of 20 µL using Dream *Taq* Green PCR Master Mix (Thermo Scientific). PCRs performed with PCR program including 2 minutes initial denaturation (94°C), denaturation for 1 min at 94°C, annealing for 30 seconds, extension for 30 seconds at 72°C with 30 cycles and final extension for 10 minutes at 72°C to produce overhang for cloning. These amplified fragments were used as PCR-AFLP markers for current analysis. Finally, genotypic frequencies produced genetic makers were investigated on each animal to observe the availability of these markers as breed explicit markers.

Statistical analysis: The probability for identification using the generated markers were calculated based on the estimated allelic frequencies against each marker to effectively appraise the efficacy of markers for differentiation between Red Sindhi and crossbred sampled populations. Arlequin software was used for Hardy-Weinberg equilibrium (HWE), likelihood ratio test for linkage disequilibrium, intra-breed haplotypes diversity and expected heterozygosity analyses (Excoffier and Lischer, 2010). Moreover, probability of identification (*Pis*) and probability of misjudgment (*Pms*) of Red Sindhi breed was calculated using different combinations of markers such as using single marker, using two markers and using all 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 Red Sindhi, *Pix* = Frequency of marker *x* in Red Sindhi population and *Piy* = Frequency of marker *y* Red Sindhi 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 $P_i = P_{if} + (1 - P_{if})P_{is}$ where, *Pi* = Combined probability of identification of Red Sindhi, *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 Red Sindhi, *Pmf* = Probability of misidentification with one marker and *Pms* = Probability of misidentification with two markers.

RESULTS AND DISCUSSION

The primary objective of present study was to develop molecular markers that can distinguish Red Sindhi cattle from crossbred population. The AFLP methodology followed was previously applied by Sasazaki *et al.* (2006).

PCR-AFLP markers: The markers based methods that had been applied to identify breeds among different food and game animal species in due course of time included AFLP in cattle (Ajmone-Marsan *et al.* 1997, Sasazaki *et al.* 2004, Sasazaki *et al.* 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 PCR in chicken (Choi *et al.* 2007) and SNP chip in cattle (Suekawa *et al.* 2010, Cooper *et al.* 2014, Cooper *et al.* 2016). However, AFLP markers were the most extensively used markers as DNA finger printing tool (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 in breed identification (Sasazaki *et al.* 2004, Negrini *et al.* 2007a, Negrini *et al.* 2007b). AFLP markers are considered to be more informative thus, were

used AFLP markers in current study as breed identification tool (Sasazaki *et al.* 2006). The detailed information about every marker used in current research i.e. primers sequences, annealing temperatures; size of product generated and information on pertinent mutations are presented in Table 2. The range for annealing temperatures for eleven markers used in current study was between 59°C and 65°C while PCR product sizes varied from 99bp to 570bp. The product sizes were different from the earlier reported analyses (Sasazaki *et al.* 2004, Sasazaki *et al.* 2006) that could be possibly explained by the difference of breeds. Insertion or deletion mutations of SNP were demonstrated by given markers. Moreover, marker size, chromosomal location and relevant gene information for each of the marker is presented in Table 3 however, the locations of LABG3 and 7 remained unidentified.

Expected heterozygosity intra-breed diversity for haplotypes: AFLP markers are informative for estimation of inbreeding and heterozygosity as reported in earlier report (Dasmahapatra *et al.* 2008). Intra-breed diversity of haplotypes and estimates of expected heterozygosity for each marker used for Red Sindhi and Crossbred cattle are presented in Table 4. Gene diversity estimates observed greater for Red Sindhi compared to crossbred cattle (0.982±0.0004 vs. 0.891±0.0004) that is clearly better than previously reported study by (Hussain *et al.* 2016). This can probably be attributed to difference of markers between current and previously reported studies (AFLP markers were used in the current study while microsatellites were used in previous report). Expected heterozygosity for LABG4 in crossbred was 0.396. However, expected heterozygosity of LABG8 was greater in crossbred than Red Sindhi (0.500 vs. 0.488) and 10 (0.430 vs. 0.077). Moreover, in Red Sindhi cattle only six markers demonstrated hetrozygosity compared to 10 markers in crossbred population. Finally expected heterozygosity of LABG2, 7 & 9 was greater in Red Sindhi than crossbred (Table 4).

Genotype and allele frequencies: Genotype and allele frequencies were estimated using AFLP-PCR and are presented in Table 5. The frequency of allele 1 ranged from 0.2 to 1.00 in Red Sindhi breed while 0.083 to 1.00 among crossbred animals whereas, frequency of allele 2 ranged from 0.00 to 0.86 against Red Sindhi and ranged from 0.00 to 0.971 against crossbred animals. Moreover, absence of PCR band as product was taken as allele 1 while presence of PCR band taken as allele 2 in the current analysis. Thus, allele 2 of LABG2, 4, 8, 10 and 11 were taken as prospective breed identification markers for Red Sindhi cattle in the current study. Similarly, allele 2 of LABG2-11 markers appeared to be crossbred specific markers. Moreover, same strategy was previously implemented to identify Japanese black cattle and crossbred populations in previous reports (Sasazaki *et al.* 2004, Sasazaki *et al.* 2006).

Table 5: Allelic frequencies of Red Sindhi and Crossbred for 11 markers.

Locus	Allelic frequency			
	Red Sindhi		Cross-bred	
	Allele1	Allele2	Allele1	Allele2
LABG1	0.780± 0.018	0.220± 0.018	1.000±0.000	0.000
LABG2	0.620 ± 0.021	0.380 ± 0.021	0.875 ± 0.014	0.125± 0.014
LABG3	1.000±0.000	0.000	0.896± 0.013	0.104± 0.013
LABG4	1.000±0.000	0.000	0.729 ± 0.019	0.271 ± 0.019
LABG5	1.000±0.000	0.000	0.646± 0.021	0.354 ± 0.021
LABG6	1.000±0.000	0.000	0.938± 0.011	0.063 ± 0.011
LABG7	0.820± 0.016	0.180± 0.016	0.083± 0.012	0.917 ± 0.012
LABG8	0.580± 0.021	0.420 ± 0.021	0.521 ± 0.022	0.479± 0.022
LABG9	0.380± 0.021	0.620 ± 0.021	0.854± 0.015	0.146± 0.015
LABG10	0.200 ± 0.017	0.800 ± 0.017	0.688 ± 0.020	0.313 ± 0.020
LABG11	1.000 ± 0.000	0.000	0.979± 0.006	0.021± 0.006

Power of Identification (*Pis*) and misjudgment (*Pms*): Each locus was tested for Hardy-Weinberg equilibrium (HWE) using genotype scores of the Red Sindhi population. No locus in the study was significantly departed from HWE at $P < 0.05$ for the animals that were tested. Likelihood ratio test applied to investigate linkage disequilibrium between any two loci and non-pairing loci gave substantial disequilibrium ($P < 0.05$). Eventually, supposition of no linkage among eleven markers provided the base for computation of identification and misjudgment probability as described by earlier researchers (Sasazaki *et al.* 2006).

Table 2: Marker information for PCR-AFLP (Vos *et al.* 1995; Malik *et al.* 2018).

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

Table 3: 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 4: Within breed diversity of haplotypes and expected heterozygosity of Red Sindhi and Cross-bred.

Breed	Diversity parameters		Expected hetrozygosity	
Red Sindhi	Sum of square freqs.	0.0200	Locus	Expected heterozygosity*
	Gene diversity	0.9818 ± 0.0004	LABG1	0.344
	Theta (Hom)	52.0 44±1.083	LABG2	0.472
	Theta (k)	13.186	LABG7	0.296
	Theta (S)	0.871± 0.384	LABG8	0.488
	Theta (Pi)	2.392± 1.441	LABG9	0.472
			LABG10	0.321
Crossbred	Sum of square freqs.	0.0208	LABG2	0.219
	Gene diversity	0.981±0.0004	LABG3	0.187
	Theta (Hom)	49.850±1.060	LABG4	0.396

Theta (k)	12.651	LABG5	0.458
Theta (S)	1.461±0.528	LABG6	0.117
Theta (Pi)	2.752±1.615	LABG7	0.153
		LABG8	0.500
		LABG9	0.250
		LABG10	0.430
		LABG11	0.041

* Results are only shown for polymorphic loci

Allele 2 was identified to use as breed identification marker using PCR technique to discriminate between Red Sindhi and crossbred populations in the country. The probability of judgment and misjudgment was calculated based on the frequency of marker LABG1, 9 & 10 in Red Sindhi and crossbred populations. These three markers showed higher frequency in Red Sindhi population whereas lower in crossbred population (Table 6). LABG10 showed probability of judgment of 0.800 and that of misjudgment of 0.313. However, combined probability of judgment for LABG1 & 9 (0.704) was lesser to LABG10 (0.800) with lesser degree of misjudgment for LABG1 & 9 (0.146). The probability of judgment of Red Sindhi was improved using all three markers (0.941) however; this also raised the misjudgment as well (0.413). Finally, a single marker was strong enough to identify Red Sindhi compared to previous study of Japanese black and crossbred cattle identification (Sasazaki *et al.* 2004, Sasazaki *et al.* 2006).

Table 6: Identification (*Pis*) and misjudgment (*Pms*) probabilities of Red Sindhi breed using different panels of markers.

Markers panel	Identification probability (*Pis*)	Misjudgment probability (*Pms*)
LABG10	0.800	0.313
LABG9	0.620	0.146
LABG1	0.220	0.000
LABG1 + 9	0.704	0.146
LABG1+9+10	0.941	0.413

Conclusions: This research generated AFLP-PCR breed specific markers to identify the purity of breed for Red Sindhi cattle. These markers may be used to continue the extension work for farmers and other stakeholders to test the purity of animals prior to entering into breeding program.

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