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Phylogenetic Relationship among Indigenous Cattle Breeds of Pakistan Based on RAPD Markers

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ABSTRACT

Genetic diversity and relationship among 10 indigenous cattle breeds (Sahiwal, Red Sindhi, Cholistani, Dajal, Dhanni, Rojhan, Lohani, Hissar, Hariana and Tharparkar) were analysed using RAPD markers. A total of 80 RAPD primers were used for PCR amplification of DNA from the cattle breeds. The PCR products were analyzed by agarose gel electrophoresis. Scoring of bands were performed to generate dendrogram using unweighted pair group method of arithmetic means (UPGMA). Six hundred and four amplified bands were observed from successful amplifications of 73 primers (average 8.2 bands per prime). Only seven primers amplified no product or unidentifiable (smeared) bands. A total of 68 polymorphic bands were observed, showing 11.25 % polymorphism on average across cattle breeds. Results revealed a low genetic variation among the cattle breeds. A RAPD primer OPF-07 was able to discriminate more than one cattle breed. Phylogenetic analysis showed the relatively high similarity indices among 10 cattle breeds (ranging from 83.85% to 89.62%). The highest similarity was observed between Dhanni and Lohani (89.62%), followed by Sahiwal and Red Sindhi with 89.44% similarity. However, the lowest similarity was observed between Cholistani and Tharparkar (83.85%). The cluster analysis of UPGMA showed two major clusters: Cluster A having Hissar and Hariana (similarity coefficient 0.87) grouping with Tharparkar; and cluster B having Dajal and Rojhan (0.88) grouped together, Dhanni clustered with Lohani while Cholistani grouped with Dajal and Rojhan. The Sahiwal breed clustered with Red Sindhi having a similarity coefficient of 0.89. The findings revealed close genetic relationships among the cattle breeds which need to be refined by an increased sample size of each breed from their respective home tracts. Moreover, further studies are warranted by using latest genomics tools to get insights into the evolution and domestication of indigenous cattle breeds.

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MSR and MSK conceived and designed the experiment. MSR and FH collected the samples and performed the experiment. FH and MSR drafted and revised the manuscript.

Key words
Diversity, Cattle, RAPD, Polymorphism, Phylogenetics

INTRODUCTION

All native cattle breeds of Pakistan belong to *Bos indicus* (zebu) species. They are well known for their adaptability under sub-tropical environment owing to their heat tolerance and tick resistance properties. Indigenous cattle breeds have been grouped into three classes including dairy breeds (Sahiwal, Red Sindhi and Cholistani), dual purpose breeds (Tharparkar, Achai and Gabrali) and draught breeds (Dajal, Bhagnari, Dhanni, Rojhan and Lohani). The cattle not only provide milk, meat and other byproducts of great value, but also play an important part as a reserve of family wealth (Payne and Wilson, 1999). Indigenous cattle are contributing significantly to milk and meat production in Pakistan where around 51.5 million cattle heads are found in a positive population trend (GOP, 2021).

Genetic improvement in performance of indigenous cattle has been sought attempted in the past by crossbreeding them with highly productive *Bos taurus* cattle breeds in different countries (Payne and Wilson, 1999). Though a substantial improvement in milk production performance through crossbreeding is observed, but such attempts have simultaneously placed some of the breeds at risk, especially in case of indiscriminate crossbreeding. At present, typical breed characteristics in many of them have been diluted, and others with unique characteristics are declining numerically (Payne and Hodges, 1997). Increasingly, these breeds will disappear unless preventive measures are taken (Alderson, 1990).

Despite significant economic, social and cultural importance of indigenous cattle breeds, genomic characterization of all cattle breeds in Pakistan has not been carried out on a large scale. Moreover, there are

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several inconsistencies and ambiguities in literature regarding the evolution of indigenous cattle breeds. For example, Cholistani are thought to have evolved from the crossing of Sahiwal with local cattle (Mason, 1996). Joshi and Phillips (1953) and Payne and Wilson (1999) stated Tharpakar as white or grey Sindhi and also claimed that Tharparkar is not a homogenous breed; rather it has the influence of Kankrej, Red Sindhi and Gir breeds. Similarly, Payne and Wilson (1999) reported that Hariana cattle are somewhat similar to the Bhagnari breed and Dajal is a light-colored small size strain of Bhagnari breed. It is, therefore, necessary to characterize and evaluate the genetic differences among breeds for their effective and meaningful management and sustainable utilization (Hammond, 2001).

Studies on the phylogenetic relationship among indigenous breeds are considered as effective tool for elucidation of the history and genetic variation among breeds. These also help to identify the footprints of human selection on cattle genomes and domestication events (Kotze and Muller, 2000).

Traditionally, external or internal phenotypic characters (skin color, size of testes, milk butterfat content, etc.) have been used for phenotypic characterization of a breed (Bradley *et al*., 1993). But phenotypic traits are affected by both genetic and environmental factors, so characterization based on phenotypes could not provide meaningful results (Baker and Manwell, 1991). Therefore, genomic characterization is the best strategy to elucidate genetic diversity and evolutionary relationships among different breeds as the latest biotechnology tools are available to analyze genome wide diversity in animal populations (Jerry *et al*., 1997). Among available DNA based tools for characterization of genetic diversity, RAPD analysis is the simplest and cheapest procedure to elucidate variations at different regions of genome (Hwang *et al*., 2001). It utilizes short random oligonucleotide primers for polymerase chain reaction (PCR) amplification of genomic DNA. Characterization of genetic variation in different animal species has been carried out by using this technique (Michelmore *et al*., 1991), especially in bovine population. Moreover, it can also be used to elucidate genetic variation within and between breeds of same species (Smith *et al*., 1996). This study aimed to characterize genetic variation among cattle breeds of Pakistan using RAPD markers.

MATERIALS AND METHODS

This study was conducted at Institute of Animal and Dairy Sciences (formerly Department of Animal Breeding and Genetics), University of Agriculture, Faisalabad and Plant Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad.

Experimental animals

Keeping in view the breed characteristics, genetically unrelated animals belonging to 10 Zebu breeds of Pakistan (Sahiwal, Red Sindhi, Cholistani, Tharparkar, Dajal, Dhanni, Rojhan, Lohani, Hissar and Hariana) were sampled from different Livestock Experiment Stations of Punjab. The sampling locations and number of samples collected per breed are presented in Table I.

Collection of blood samples

The peripheral blood was collected from the selected animals of each breed in EDTA coated vacutainers under hygienic conditions. Blood samples were stored at –20 °C until DNA extraction.

DNA extraction and quantification

Previously reported phenol-chloroform based method was used to extract genomic DNA from blood (Singer *et al*., 1988; Grimberg *et al*., 1989). The DNA quantification was performed using DyNA Quant fluorometer (Hoefer, Inc., San Francisco, CA). Extracted DNA samples were run on 0.8 % agarose gel to observe quality of extracted DNA and samples with smears were rejected.

PCR amplification using RAPD analysis

To carry out PCR amplification, concentration of DNA, 10X PCR buffer with $(NH_4)_2SO_4$, $MgCl_2$, dNTPs (dATP, dCTP, dGTP and dTTP), 10 Mer random primer and *Taq* DNA polymerase were optimized (Williams *et al*., 1990). For PCR amplification, random primers (10 bp) from Operon Company (Operon Tech. Inc., Alameda, CA, USA) were used.

Bulked segregant analysis (BSA)

The DNA samples from each breed were pooled together by adding an equal volume of DNA from individual samples (number of samples used from each breed are mentioned in Table I). PCR was carried out in 25 µl reaction mixture containing 3 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 uM primer, 5 ng of genomic DNA and 1 unit of *Taq* polymerase through programmable thermal cycler (Eppendorf, USA) by using 80 RAPD primers. The thermocycling profile for RAPD was initial denaturation at 94 °C for 5 min, followed by 1 min denaturation at 94 °C, primer annealing at 36 °C for 1 min and extension at 72 °C for 1 min for 40 cycles, and then a final extension at 72 °C for 10 min.

The PCR products were analyzed through agarose gel electrophoresis (1.2%) using ethidium bromide staining. Bands were analyzed by the ultraviolet trans-illuminator and photographed by using the stratagene eagle eye still video system.

Table I. Location, number of animals sampled and phenotypic description of 10 cattle breeds used in the present study.

Breed	Location	Number	Description	
			Type	Color
Sahiwal	University of Agriculture, Faisalabad	5	Dairy	Reddish Brown
	LES* Khizerabad	20		
	LES Kallurkot	10		
	LES Fazilpur	5		
Red Sindhi	LES 205/TDA	20	Dairy	Deep red
	BLPRI Kherimurat	20		
Cholistani	LES Jogaitpeer	21	Dairy	White coat splashed with black/ brown spots
Dajal	BLPRI** Kherimurat	10	Draught	White / Grey
Dhanni	BLPRI Kherimurat	20	Draught	White coat with black/red/brown spots
Rojhan	Rojhan Tehsil District Rajanpur	7	Draught	Red and White spotted coat
Lohani	BLPRI Kherimurat	20	Draught	Red coat splashed with white spots
Hissar	LES Kallurkot	6	Draught	Greyish black
Hariana	LES Kallurkot	6	Draught	Grey white
Tharparkar	LES Rakh Mahni	10	Dual	Grey white

*Livestock Experiment Station; ** Barani Livestock Production Research Institute.

Scoring and analysis of the data

Analysis of electrophoretic profiles of each breed was carried out by scoring the number of amplified DNA bands as present (1) or absent (0). These profiles for each primer from 10 cattle breeds were compared with each other to elucidate genetic similarities. Number of shared bands produced by each primer in different breeds were used to determine genetic relationship by using following equation (Nei and Li, 1979).

$$F = 2N_{xy} / (N_x + N_y)$$

Where, F is the similarity coefficient, N_x and N_y are the numbers of fragments in breeds x and y, respectively, whereas N_{xy} is the shared fragments in the breeds. These similarity coefficients were employed to make dendrogram (phylogenetic tree) by using unweighted pair group method of arithmetic means (UPGMA) (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

Optimization of PCR conditions

For RAPD analysis, it is very important to optimize the PCR conditions as many factors can influence the reproducibility of PCR reactions like primer sequence, quantity and quality of template DNA, the type of *Taq* DNA polymerase and thermal cycler (Penner *et al*., 1993). Reproducible RAPD patterns could be obtained by using standard PCR reaction conditions. In this study, DNA concentration of 5 ng/25 µL was optimized as suitable to produce most consistent results regarding amplified fragments. Similarly, 3 mM concentration of $MgCl_2$ and 1 unit/25 L reaction of *Taq* DNA polymerase were observed to produce most consistent amplified products. Other PCR reaction conditions were also kept to be consistent and reproducible.

Polymorphism of the RAPD markers among indigenous cattle breeds

A total of 80 RAPD primers were used to amplify DNA from 10 indigenous cattle breeds. Only seven primers (OPD-02, OPD-12, OPD-14, OPD-16, OPD-17, OPH-10 and OPO-17) failed to amplify any fragment or produce clear bands. The remaining 73 primers amplified 604 fragments, yielding an average of 8.2 bands per primer and a range between two (OPF-11) and 19 (OPF-08) (Table II), which were similar with earlier studies (Chen *et al*., 2001; Ramesha *et al*., 2002). DNA polymorphism revealed by the 73 primers was around 11.25% (68/608 bands) among the 10 cattle breeds.

Our study concluded that one breed specific RAPD primer or a combination of two or more markers can be utilized to recognize indigenous cattle breeds. We identified one primer (OPF-07) that was able to identify more than one breed (Fig. 1). In this study, Lohani breed produced the highest number of amplified bands (454) while the lowest (432) was observed in Tharparkar.

Genetic similarity and phylogenetic analysis

Similarity coefficients calculated on the basis of shared amplified bands in different breeds were used to generate similarity matrix of the 10 cattle breeds (Nei and Li, 1979) and presented in Table III. Overall similarity coefficients of indigenous breeds were high (ranging from 83.85% to 89.62 %). The highest similarity (89.62%) was observed between Dhanni and Lohani sharing 449 out of the 454 total bands. The second highest similarity (89.44%) was found between Sahiwal and Red Sindhi sharing 407 out of 437 total bands. The lowest similarity (83.85%) was observed between Cholistani and Tharparkar. Tharparkar showed the least genetic similarity with all other breeds.

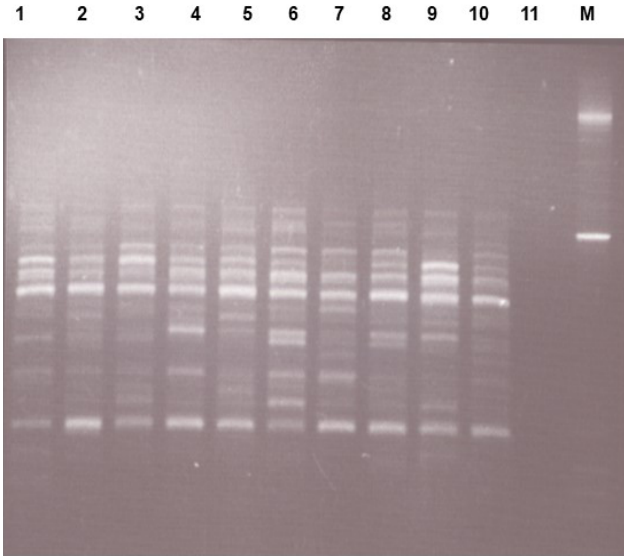

Fig. 1. Amplification profile of 10 cattle breeds with primer OPF-07.
Lane 1: Sahiwal; Lane 2: Red Sindhi; Lane 3: Cholistani; Lane 4: Dajal; Lane 5: Dhanni; Lane 6: Rojhan; Lane 7: Lohani; Lane 8: Hissar; Lane 9: Hariana; Lane 10: Tharparkar; Lane 11: negative control and Lane M: size marker.

Phylogenetic tree among indigenous cattle breeds was generated by using the similarity coefficients employing UPGMA method (Fig. 2). Genetic diversity in these cattle breeds was low as revealed by very high similarity coefficients. The cluster analysis of similarity matrices of the 10 indigenous cattle breeds revealed two main groups. Cluster 'A' consisted of three cattle breeds (Hissar, Hariana and Tharparkar). Cluster 'B' was further divided into three subclusters 'b_1', 'b_2' and 'b_3'. The subcluster 'b_1' consisted of Dajal, Rojhan and Cholistani. The pair of a closely related breeds (Dhanni and Lohani) was found in the sub-cluster 'b_2'. The sub-cluster 'b_3' had Sahiwal and Red Sindhi.

Phylogenetic analysis showed the relatedness among the Pakistani cattle genetic resources, which indicated their common ancestry and admixture. Present day domestic zebu cattle is believed to be originated from the extinct aurochs, *Bos primigenius* (Payne, 1991; Troy *et al*., 2001). Sub-clustering indicates the gene flow that might be due to haphazard crossbreeding among different breeds in their home tracks due to lack of execution of the provincial and national breeding policies in the country.

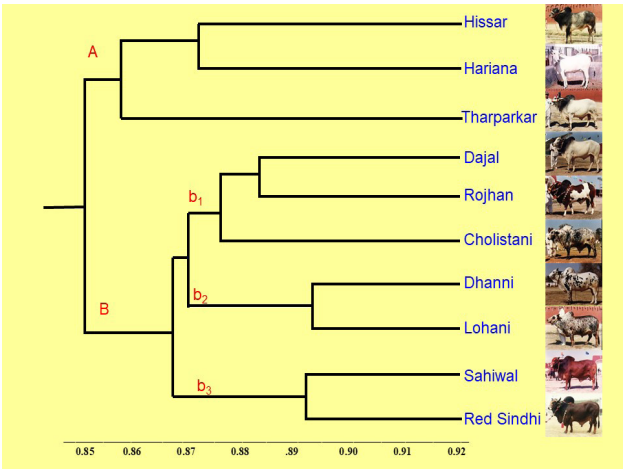

Fig. 2. A UPGMA dendrogram showing phylogenetic relationship among 10 cattle breeds of Pakistan.

In agreement to our results, the genomic comparison of SNPs of five important diverse zebu cattle breeds of Pakistan revealed a close relationship between Sahiwal and Red-Sindhi sharing 173,344 SNPs (Iqbal *et al*., 2019).

Our results are contrasted from the evaluation of Pakistani indigenous cattle breeds using microsatellite markers, which indicated the highest genetic similarity between Tharparkar and Red Sindhi, followed by that between Dhanni and Sahiwal, and then between Lohani and Achai, while Dajal breed was placed at a distinct position (Hussain *et al*., 2016).

A comparative study on microsatellite and RAPD markers in camel revealed that microsatellites markers are powerful tools for genetic characterization of breeds while RAPD markers are effective to identify genetic variation within and between breeds and to reveal the genetic relatedness in many livestock breeds (Mahrous *et al*., 2011). Our findings are also consistent with the phenotypic and historic relatedness of Pakistani cattle breeds. Further comprehensive genomic studies are required for the molecular characterization of Pakistani indigenous cattle breeds in terms of evolution and genome wide diversity.

Table II. RAPD primers amplified, and total number of fragments scored for each primer.

S. No.	Primer code	No. of amplified loci
1	OPD-01	11
2	OPD-03	7
3	OPD-04	7
4	OPD-05	9
5	OPD-06	7
6	OPD-07	7
7	OPD-08	6
8	OPD-09	5
9	OPD-10	3
10	OPD-11	5
11	OPD-13	5
12	OPD-15	6
13	OPD-18	7
14	OPD-19	5
15	OPD-20	6
16	OPE-01	8
17	OPE-02	10
18	OPE-03	6
19	OPE-04	8
20	OPE-05	7
21	OPE-06	12
22	OPE-07	8
23	OPE-08	8
24	OPE-09	14
25	OPE-10	9
26	OPF-01	8
27	OPF-02	6
28	OPF-03	13
29	OPF-04	13
30	OPF-05	6
31	OPF-06	15
32	OPF-07	16
33	OPF-08	19
34	OPF-09	9
35	OPF-10	8
36	OPF-11	2
37	OPF-12	4
38	OPH-01	10
39	OPH-02	9
40	OPH-03	11
41	OPH-04	10
42	OPH-05	9
43	OPH-06	10
44	OPH-07	8
45	OPH-08	12
46	OPH-09	9
47	OPI-01	8
48	OPI-02	6
49	OPI-03	6
50	OPI-04	11
51	OPI-05	4
52	OPI-06	15
53	OPI-07	11
54	OPI-08	9
55	OPM-03	6
56	OPM-13	9
57	OPM-18	12
58	OPM-19	6
59	OPO-02	9
60	OPO-16	5
61	OPO-19	5
62	OPQ-06	10
63	OPQ-11	5
64	OPQ-16	6
65	OPQ-19	9
66	OPR-01	9
67	OPR-02	6
68	OPR-03	4
69	OPR-10	8
70	OPT-03	4
71	OPT-07	12
72	OPT-09	7
73	OPT-18	9

CONCLUSIONS

Genetic diversity among Pakistani indigenous cattle breeds is very low and breed clustering pattern based on RAPD markers is consistent with their phenotypic and historic relatedness. Sampling of pure specimens from the home tracts of respective breeds with authentic pedigree records is suggested. Moreover, recent genomics tools like SNP genotyping and whole genome re-sequencing should be used to refine our findings among indigenous cattle breeds of Pakistan.

Table III. Similarity matrix of 10 cattle breeds obtained from RAPD markers.

	1	2	3	4	5	6	7	8	9	10
1. Sahiwal	1									
2. Red Sindhi	0.8944	1								
3. Cholistani	0.8716	0.8787	1							
4. Dajal	0.8553	0.8688	0.8777	1						
5. Dhanni	0.8763	0.8810	0.8627	0.8757	1					
6. Rojhan	0.8620	0.8785	0.8813	0.8879	0.8764	1				
7. Lohani	0.8597	0.8681	0.8751	0.8713	0.8962	0.8929	1			
8. Hissar	0.8625	0.8498	0.8664	0.8633	0.8541	0.8696	0.8732	1		
9. Hariana	0.8404	0.8618	0.8518	0.8568	0.8562	0.8648	0.8587	0.8775	1	
10.Tharparkar	0.8565	0.8704	0.8385	0.8511	0.8661	0.8626	0.8611	0.8543	0.8705	1

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Statement of conflict of interest

The authors have declared no conflict of interest.

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