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Microsatellite Markers Based Genetic Evaluation of Pakistani Cattle Breeds

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ABSTRACT

Animal Genetic Resource of Pakistan is very diverse. There are 15 distinct breeds of cattle in Pakistan all belonging to zebu (humped type) cattle (*Bos indicus*) for which very little information on genetic architecture is available. Microsatellite markers are being widely used for breed characterization in animals. In the present study 345 individuals of 11 breeds (10 Pakistani cattle breeds and exotic Holstein Friesian breed) were genotyped using 21 fluorescently–labeled microsatellite markers to assess genetic variation and relationships among them. All markers were polymorphic in all studied breeds and observed number of alleles ranged from 6.28 in Tharparker to 10.66 in Dajal with mean value 8.50±1.552. Average values of observed and expected heterozygosity were calculated as 0.4897±0.1538 and 0.8292±0.0744 respectively. Mean values of Fis, Fit, F_{ST} and gene flow were 0.2819, 0.3864 and 0.1456 respectively. The average PIC value was 0.81 showing suitability of these markers for forensic analyses. Nei's genetic distance estimates indicated relatively close genetic identity between Tharparker and Red-Sindhi breeds of Sindh Province of Pakistan while Tharparker and Dajal breeds of Pakistan were found most distinct. The UPGMA-based phylogenetic tree constructed from the genetic distances also indicated that the cattle breeds of Pakistan can be classified into distinct genetic groups based on these markers. This is the first comprehensive report on molecular characterization of Pakistani cattle breeds using microsatellite markers. This study can be helpful for making breed conservation strategies of cattle in Pakistan in future.

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Authors' Contribution
TH and MEB conceived and designed the study. TH, AW, AA did sampling and genome extraction. TH, AA, ZA amplified the markers. TH, SOP, AW, KK, MDD analyzed the data. TH, MEB, MW, SOP, AA, MDD, IGI wrote the article.

Key words
Microsatellite markers,
Heterozygosity,
Pakistani cattle breeds

INTRODUCTION

There is a great animal genetic diversity in Pakistan due to its wide range of geographical ad environmental diversity strengthening its agricultural economy. There are 39.6 million cattle with 15 distinct cattle breeds located across the country raised mainly for milk, meat and draft purposes (Anonymous, 2012). All the indigenous Pakistani cattle belong to zebu (humped type) cattle (*Bos indicus*). The classification of the native cattle breeds of Pakistan has traditionally been based on differences in phenotypic features but these differences are insufficient to identify them distinctly. Characterization and evaluation of genetic differences among these breeds is necessary for their effective and meaningful improvement and conservation. A number of techniques have been used to study genetic diversity and molecular phylogeny of domestic animals. Microsatellite markers, due to their co-dominant and multi-allelic attributes, have been proven to be useful markers for a variety of purposes, such as genome mapping, determination of genetic variation, parentage, assessment of within and among breeds genetic diversity and inbreeding levels, introgression from other species, admixture among breeds evaluating differences within of cattle and determining population substructure (Ciampolini *et al*., 1995; Garcia–Moreno *et al*., 1996; MacHugh *et al*., 1998; Edwards *et al*., 2000; Canon *et al*., 2001; Tapio *et al*. 2006; Ginja *et al*. 2009; Li and Kantanen, 2009; Qi *et al*., 2009).

The present study was designed to characterize 9 famous indigenous cattle breeds of Pakistan (Sahiwal, Cholistani, Red-Sindhi, Tharparker, Dhanni, Lohani, Dajal, Bhagnari, Achai) along with Nari-Master breed (a recently developed beef breed by crossing Bhangnari and Australian Drought Master) and exotic Holestein Friesian

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Table I.- Summary statistic for population genetic parameters for eleven studied breeds obtained averaging over all 21 microsatellite markers. All breeds along with species (*Bos indicus, Bos taurus* and Hybrid); type; location; number of samples; alleles richness; observed heterozygosity; expected heterozygosity; Fis, inbreeding coefficient and Garza-Williamson index.

Breed	Species	Type	Location	Samples	AR_SD	Ho_SD	He_SD	Fis	G-W index
Sahiwal	*Bos indicus*	Dairy	Punjab	40	9.9524 (3.3982)	0.4810 (0.2482)	0.7748 (0.0964)	0.3714	0.4530
Cholistani	*Bos indicus*		Punjab	36	8.1905 2.6574)	0.5066 (0.2487)	0.6695 (0.1570)	0.2326	0.4880
Red Sindhi	*Bos indicus*		Sindh	20	6.7619 (3.1766)	0.5381 (0.2743)	0.6571 (0.1888)	0.1602	0.3990
Tharparker	*Bos indicus*		Punjab, Balochistan	24	6.1429 (3.2293)	0.5218 (0.2898)	0.6017 (0.2238)	0.1142	0.4060
Dhanni	*Bos indicus*	Dual purpose	Sindh	25	7.7143 (3.4080)	0.4343 (0.2993)	0.7148 (0.1660)	0.3800	0.5320
Lohani	*Bos indicus*	Draught	Punjab	23	7.0476 (3.3388)	0.4969 (0.2789)	0.6642 (0.1931)	0.2352	0.4660
Dajal	*Bos indicus*		Punjab, Khyber Pakhtunkhwa	40	11.1429 (4.2460)	0.4595 (0.2854)	0.7778 (0.1394)	0.4018	0.6330
Bhagnari	*Bos indicus*		Punjab	31	8.1905 (2.9089)	0.4314 (0.2034)	0.6992 (0.1549)	0.3725	0.5080
Achai	*Bos indicus*		Balochistan	31	9.6190 (3.8403)	0.5760 (0.1873)	0.7228 0.1456)	0.1900	0.4940
Nari Master	*Hybrid*	Beef	Khyber Pakhtunkhwa	50	10.2858 (4.0883)	0.5257 (0.2265)	0.7355 (0.1160)	0.2780	0.5590
Holstein Friesian	*Bos taurus* (exotic)	Dairy	Punjab, Balochistan	25	7.7143 (2.9179)	0.4629 (0.2894)	0.6945 (0.1889)	0.3199	0.5250

present in the country, at molecular level using a set of 21 fluorescently labeled microsatellite markers for the analysis of within and between breeds genetic variability.

MATERIALS AND METHODS

Blood sample collection and DNA extraction

Blood samples (10 ml) were collected aseptically during extensive travelling to respective breeding areas of each selected cattle breed in all four provinces of Pakistan from phenotypically pure animals. Sampling from related individuals was avoided to keep them unrelated (Table I). EDTA added blood was kept in ice and transported to Molecular Biology and Genomics Lab of Institute of Biochemistry & Biotechnology of University of Veterinary and Animal Sciences, Lahore for DNA extraction following protocol used by Babar *et al*. (2009).

Microsatellite markers and genotyping

Twenty one microsatellites markers (BM1818, BM1824, BM2113, BM6526, CSSM66, ETH10, ETH225, HAUT27, ILSTS011, ILSTS029, ILSTS033, LSTS044, ILSTS049, ILSTS052, INRA005, INRA32, INRA63, TGLA122, TGLA126, TGLA227, INRA023) (Fluorescently labeled with PET, NED, VIC, HEX, 6-FAM) were selected from the International Society for Animal Genetics and Food and Agriculture Organization of the United Nations (ISAG and FAO) working group (FAO, 2004) recommended markers be used on the DNA samples of all famous cattle breeds to establish specific genotypes (Table II). All markers were amplified in all samples by multiplex PCR and genotyped on ABI Prism 3130XL genetic analyzer (Applied Biosystems, Inc., Foster City, CA) using the GeneScan™ -500 LIZ® as Size Standard.

Statistical analysis

Genotyping data was analyzed for calculation of different population genetics parameters for within–breed genetic diversity like observed number of alleles (Na), effective number of alleles (Ne) for each polymorphic locus, within–population inbreeding estimate also known as Wright's (1978) fixation index (Fis), Shannon's Information index (I), observed heterozygosity (Ho), expected heterozygosity (He), Garza-Williamson index (G-W index), F statistics (Fis, Fit, Fst), Nei's measures of genetic identity and distance (Nei, 1972), and gene flow (Nm), Hardey-Weinberg equilibrium (HWE) at each locus for each breed. Dendrogram, and AMOVA (Analysis of Molecular Variance) analysis was done to calculate genetic identity and genetic distance among

Table II.- Over all summary of 21 microsatellite marker loci consolidating all breeds and all animals: number of alleles, effective number of allele, observed heterozygosity, expected heterozygosity, Garza-Williamson state, F-statistic (Fis, Fit and Fst) and polymorphic information content.

Locus	na	ne	Ho	He	G.W stat.	Fis	Fit	Fst	PIC
BM1818	21	7.6580	0.4841	0.8707	1.0000	0.3983	0.4575	0.0984	0.86
BM1824	16	4.9731	0.5942	0.8001	0.2424	0.1300	0.2331	0.1185	0.78
BM2113	21	8.0338	0.7101	0.8768	0.3650	0.0943	0.1838	0.0988	0.86
BM6526	40	15.0019	0.6464	0.9347	0.8888	0.1706	0.2863	0.1395	0.93
CSSM66	24	2.3574	0.7478	0.5766	0.3846	-0.3657	-0.2884	0.0566	0.84
ETH10	23	9.0424	0.4319	0.8907	0.6969	0.4018	0.5117	0.1837	0.88
ETH225	25	3.7459	0.5275	0.7341	0.3906	0.1888	0.2731	0.1040	0.72
HAUT27	29	4.0666	0.3565	0.7552	0.8787	0.3593	0.5025	0.2235	0.73
ILSTS011	20	7.9281	0.2638	0.8751	0.9090	0.6681	0.7041	0.1084	0.86
ILSTS029	18	9.7999	0.3130	0.8993	0.5142	0.5126	0.6334	0.2478	0.89
ILSTS033	16	4.9257	0.5043	0.7981	0.5357	0.2456	0.3407	0.1261	0.77
ILSTS044	15	3.2138	0.2986	0.6898	0.6250	0.4218	0.5466	0.2158	0.66
ILSTS049	11	4.2924	0.5449	0.7681	0.3142	0.0641	0.2598	0.2091	0.75
ILSTS052	23	7.2064	0.5043	0.8625	0.6764	0.3117	0.3777	0.0959	0.85
INRA005	21	6.2913	0.5971	0.8423	0.9545	0.1642	0.2569	0.1109	0.82
INRA32	10	2.8576	0.0638	0.6510	0.9090	0.8943	0.9040	0.0917	0.59
INRA63	20	5.2604	0.4232	0.8111	0.4651	0.4269	0.5329	0.1849	0.79
TGLA122	26	11.4107	0.5188	0.9137	0.8387	0.3447	0.4350	0.1379	0.91
TGLA126	43	10.4376	0.6464	0.9055	0.6615	0.1657	0.2590	0.1119	0.90
TGLA227	21	5.9704	0.6029	0.8337	0.6363	0.0861	0.2816	0.2139	0.82
INRA023	26	6.8922	0.6000	0.8561	0.7428	0.1954	0.3245	0.1605	0.83
Mean	22.3333	6.7317	0.4943	0.8164	0.6490	0.2819	0.3864	0.1456	0.81
St. Dev	7.9771	3.1685	0.1647	0.0930	0.2313				

breeds. POPGENE (Yeh *et al.,* 1999) program version. 3.3 and Arlequin version 3.5.1.3 (Excoffier and Lischer, 2010) packages were used for these analyses.

A model-based Bayesian clustering analysis was used to infer population structure and the level of admixture in the Cattle breeds implemented in STRUCTURE v 2.3 (Pritchard et al. 2000). The STRUCTURE algorithm assumes K populations, each of which is in Hardy-Weinberg and Linkage equilibria, characterized by a set of allele frequencies at each locus. Analysis was performed with a burn-in length of 20,000 followed by 100,000 Markov chain Monte Carlo iterations for each K = 1 to 11 with 10 runs for each K using independent allele frequencies and an admixture model. Results across ten runs at each K were compared based on similarity coefficient (SC) as previously described in Pritchard et al. (2000). The breeds were assigned a wide cluster based on major ancestry and submitted to a second round of STRUCTURE analysis performed within each wide cluster. This method was previously described by Agaviezor et al. (2012)

RESULTS

All of 21 microsatellite markers were successfully amplified in all selected 11 breeds. All markers were polymorphic. A total of 476 alleles were identified in all 11 breeds across 21 markers ranging from 10 (INRA32) to 43 (TGLA126) alleles per locus, with an overall mean number of alleles of 22.667±8.181. The effective number of alleles ranged from 2.3574 (CSSM66) to 15 (BM6526) with mean value of 6.7317±3.1685. The Shannon's Information index value ranged from 1.3431 (INRA32) to 3.0908 (BM6526) and mean value was calculated as 2.2168±0.4612. The observed heterozygosity was ranging from 0.0753 (INRA32) to 0.7188 (BM2113) while expected heterozygosity ranged from 0.6519 (INRA32) to 0.9348 (BM6526). Means for observed and expected heterozygosities were 0.4897±0.1538 and 0.8292±0.0744 respectively.

The F statistics estimate ($F_{IS,}$ F_{IT} and Fs_T) ranged between -0.3657, -0.2884 and 0.0566 (CSSM66) to 0.8943, 0.9040 (INRA32) and 0.2478 (ILSTS029) respectively. The mean values of F_{IS}, F_{IT} and F_{ST} were 0.2819, 0.3864 and 0.1456 respectively. The value of gene flow (Nm) ranged from 0.7589 (ILSTS029) to 4.1636 (CSSM66) with mean value 1.4676. The polymorphism information content (PIC), an parameter indicative of the degree of informativeness of a marker was ranging from 0.59 (INRA32) to 0.93 (BM6526) while average value was calculated as 0.81 (Table II).

Genetic variation within-breeds

Supplementary Table I shows the within-breed genetic diversity estimates in all studied cattle breeds. The lowest observed and effective number of alleles was found 6.28 and 3.33 in Tahrparker while highest values were 10.66 and 5.70 in Dajal breed respectively. The observed heterozygosity was lowest 0.408 in Dajal while highest 0.575 in Achai cattle. Tharparker breed was having lowest (0.614) expected heterozygosity while Sahiwal breed was having highest (0.795) value. Garza-Williamson index ranged from 0.399 in Red-Sindhi to 0.559 in Nari-Master cattle.

Genetic relationship between breeds

The highest genetic distance (0.20409) was observed between Tharparker and Dajal local breeds of Pakistan but overall Tharparker and Friesian cattle breeds were found the most distinct from each other in this study with genetic distance of 0.22009. The lowest genetic distance 0.02445 was observed between Tharparker and Red-Sindhi cattle breeds (Table III). The AMOVA analysis revealed that there is 14.05% variation among studied breeds while 85.95% variation exists within breeds (Table IV).

The dendrogram based Nei's (1972) genetic distance UPGMA method modified from NEIGHBOR procedure of PHYLIP Version 3.5 indicated highest genetic similarity between Tharparker and Red-Sindhi and distinct position of Dajal breed (Fig. 1). The exotic Friesian and cross bred Nari-Master also showed high similarity.

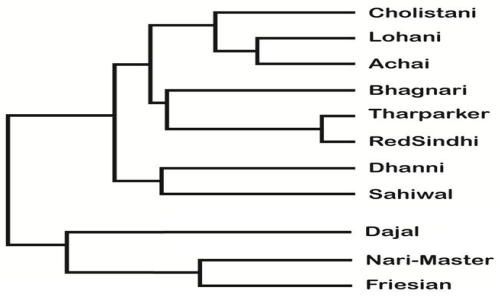


Fig. 1. Dendrogram based Nei's (1972) genetic distance: UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5

Results from STRUCTURE analysis revealed varying number of presumed ancestral populations (K) produced clusters that are similar to the information provided by the Dendogram. The first level of clustering (K=2) reflects the presence of two clusters in the 11 breeds evaluated. Further evaluation using K=3 …..K=11separated the breed into different sub ancestral

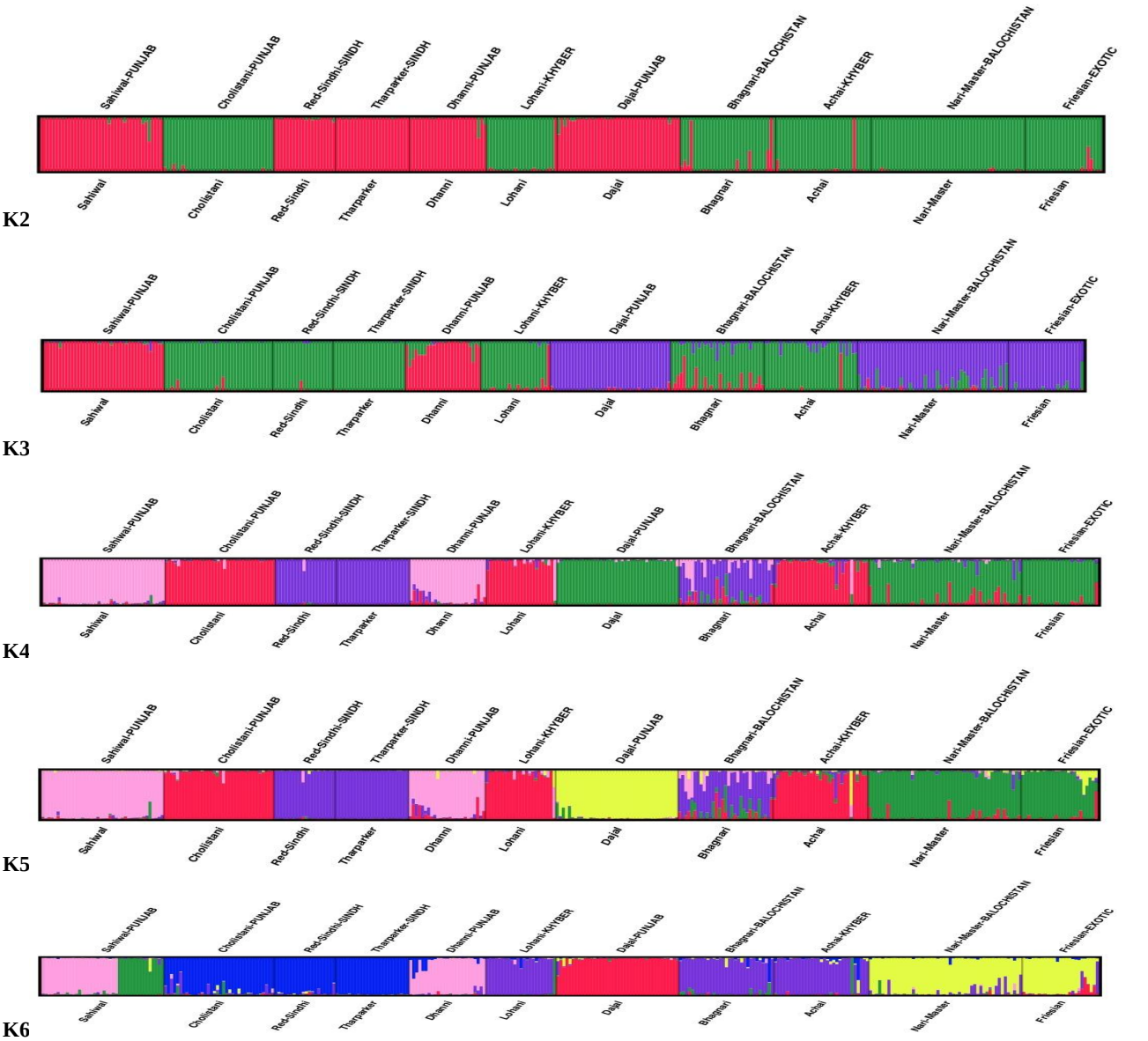
Table III.- Distance method: No. of different alleles (F_{ST}).

	Sahiwal	Cholistani	Red-Sindhi	Tharparker	Dhanni	Lohani	Dajal	Bhagnari	Achai	Nari- Master	Friesian
Sahiwal	0										
Cholistani	0.15941	0									
Red-Sindhi	0.13927	0.13612	0								
Tharparker	0.15343	0.15319	0.02445	0							
Dhanni	0.09122	0.13495	0.12434	0.14789	0						
Lohani	0.11309	0.10000	0.13041	0.1312	0.12717	0					
Dajal	0.13487	0.19776	0.17886	0.20409	0.16923	0.18211	0				
Bhagnari	0.10906	0.15787	0.11813	0.1282	0.13216	0.11099	0.16752	0			
Achai	0.10322	0.08974	0.09467	0.11185	0.1054	0.05227	0.14398	0.10392	0		
Nari Master	0.10145	0.17251	0.16662	0.17898	0.15641	0.13508	0.13698	0.10203	0.09369	0	
Friesian	0.16352	0.20863	0.20396	0.22009	0.19561	0.17957	0.12336	0.17847	0.13977	0.10682	0

Table IV.- AMOVA design and result.

	d.f.	Sum of Squares	Variance components	Percentage of variation
Among Populations	10	806.555	1.17999	14.05
Within Populations	679	4903.053	7.22099	85.95
Total	689	5709.609	8.40098	
Fixation Index	F_{ST}	0.14046		

Weir and Cockerham, 1984; Excoffier *et al.*, 1992; Weir, 1996.



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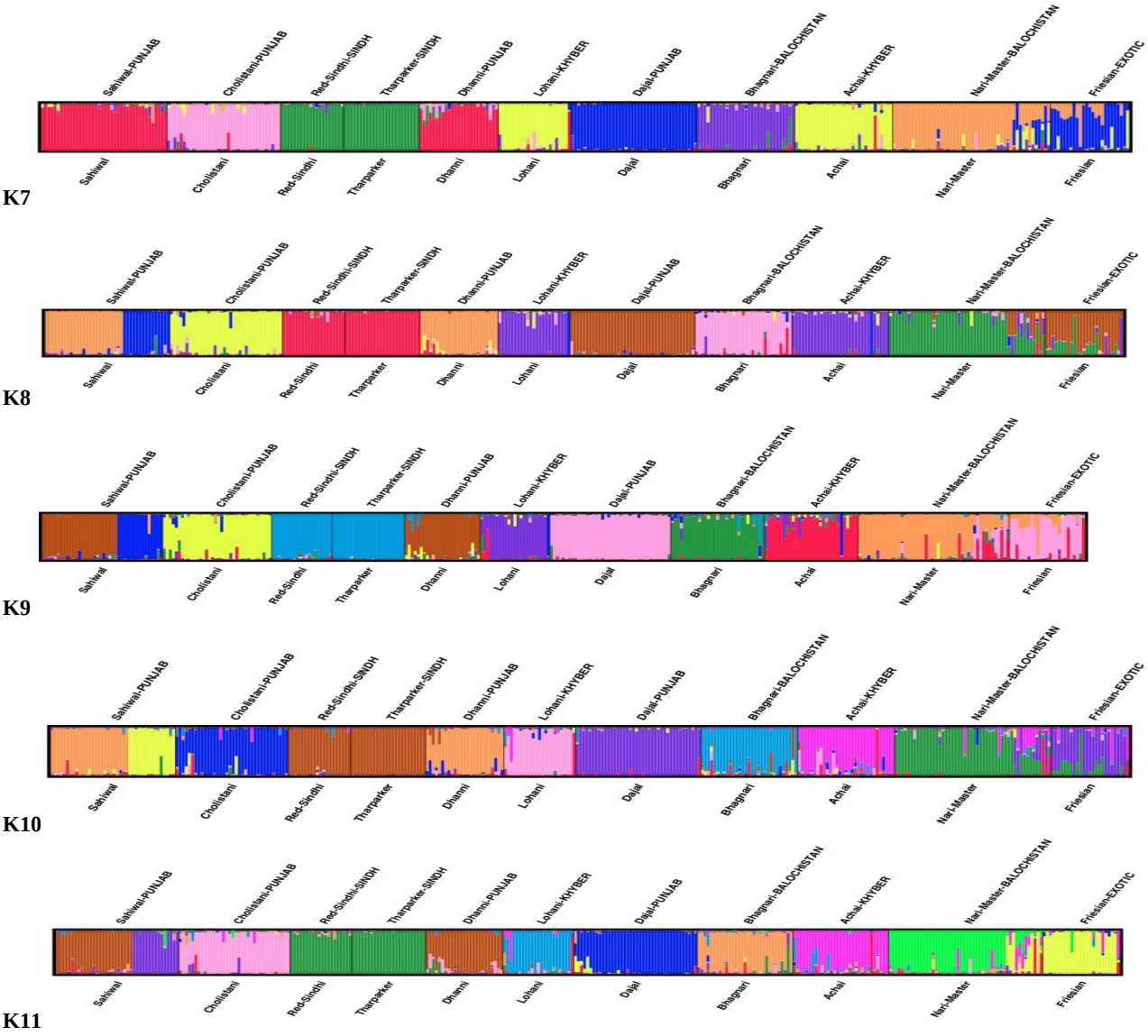

Fig. 2. Cattle population structure assessed by Structure Bar plot, generated by DISTRUCT, depicts classifications with the highest probability under the model that assumes independent allele frequencies and inbreeding coefficients among assumed clusters. Each individual is represented by a vertical bar, often partitioned into colored segments with the length of each segment representing the proportion of the individual's genome from K = 2, 3, …, or 11 ancestral populations. Breeds are separated by black lines.

groups with significant mixing between theses breeds (Fig. 2). At K=11, Red Sindhi and Tharparker breed clustered together and the same results was obtained for Sahiwal and Dajal.

DISCUSSION

To the best of our knowledge this is the first large scale report on the genetic diversity and differentiation of cattle breeds of Pakistan. The genotype data showed the genetic variation in the local Pakistani cattle populations and studied breeds could be considered as divergent genetic entities. The ten local breeds and one exotic (Holstien Friesian) were considered for genetic diversity within and between them using 21 microsatellite markers. The results showed that the Pakistani cattle populations exhibit a comparatively high level of genetic variation as estimated by allelic diversity and heterozygosity. Some

breeds such as Dajal (*Bos indicus*) and Nari Master (*Bos indicus* x *Bos taurus*) had the highest genetic diversity, whereas Tharparker and Red Sindhi showed the lowest genetic diversity between them. Significant correlations were found between Dajal and Nari Master, especially with allelic richness measure. They are genetically distinct from other Pakistani breeds and contribute significantly to the overall genetic diversity. Whereas the Tharparker and Red Sindhi breeds are somewhat isolated and raised in adjoining areas of Sindh province. So due to their remoteness from main cattle breeding areas and cross breeding the level of genetic diversity is least between them as compared to other cattle breeds of Pakistan.

The total number of alleles per locus ranged from 10 (INRA32) to 43 (TGLA126) with a mean value of 22. 3333±7.9771, indicating that all the microsatellite loci were sufficiently polymorphic and were pertinent to analyze genetic diversity. The mean number of allele (MNA) was significantly higher than Colombian cattle breeds 14.2 (Montoya *et al.,* 2010), Brazillian cattle breeds (MNA = 12) (Egito *et al.,* 2007), Argentine and Bolivian Creole cattle breeds (MNA = 7.2) (Liron *et al.,* 2006), Portuguese cattle breeds (MNA = 8.3) (Ginja *et al.,* 2010), Latine America Creole cattle (MNA = 6.2) (Delgado *et al.,* 2012), Brazilian Nellore cattle (MNA = 9.4) (Cervini *et al.,* 2006). The high allelic diversity observed is perhaps due to no selection pressure for the development of certain traits such as draught characters. The Dajal breed showed high allelic diversity (11.1429±4.2460) and Tharparker showed lowest (6.1429±3.2293), with little difference among the remaining cattle breeds fluctuating around 8.19. However, high allelic diversity was found in Nari Master (10.2858±4.0883), which is hybrid cattle breed of crossed between Bhagnari (*Bos indicus)* and Australian draught master *(Bos taurus*) and Sahiwal cattle breed also showed mean value of more than 10 alleles per locus.

Heterozygosity is an appropriate measure of genetic variability within a population when populations are expanding (Hanslik *et al.,* 2000). The observed heterozygosity ranged from 0.0638 (INRA32) to 0.7478 (CSSM66) while the range for expected heterozygosity found to be ranging from 0.5766 (CSSM66) to 0.9347 (BM6526) with average mean of 0.4943±0.1647 and 0.8164±0.0930 respectively. Among eleven breeds the Achai breed showed highest genetic diversity 0.5760±0.1873 while lowest genetic diversity was found in Bhagnari 0.4314±0.2034. Using 30 microsatellite loci, Mateus *et al.* (2004) found average observed heterozygosity ranging from 0.5533 to 0.7430 in 10 native Portuguese cattle breeds, American Charolais and the Brazilian Caracu. Egito *et al.* (2007) by using 22 microsatellite markers found average observed heterozygosity ranging from 0.6316±0.0146 (Jersey) to 0.7409±0.0097 (Mocho Nacional) in 10 Brazilian cattle breeds. Tharparker and Red Sindhi breeds showed very similar pattern of heterozygosity might be of much closed demographically. Pakistani Tharparker cattle breed showed slightly lower heterozygosity level (0.5218±0.2898) than Indian Tharparker cattle breed (0.5700), also slight genetic diversity difference was observed between Pakistani Sahiwal (0.4810) and Indian Sahiwal cattle breed (0.4200).

The Polymorphic Information Content (PIC) is a parameter indicative of the degree of informativeness of the microsatellite markers. The PIC of the polymorphic loci ranged from 0.59 (INRA32) to 0.93 (BM6526) with mean value 0.81. According to the selection standard, microsatellite markers ought to have at least 4 alleles to be considered useful to evaluate the genetic diversity. Dajal breed showed the highest average PIC value (0.74) and the Tharparker breed showed lowest (0.59). These finding are in accordance with the findings of Egito *et al.* (2007), who used 22 microsatellite markers and obtained PIC mean value 0.74.

Two Pakistani breeds, Tharparker and Red Sindhi showed the lowest inbreeding coefficient (F_{IS}) among all eleven studied cattle breeds. The dispersion of these populations in the Thar Desert region of Sindh with very harsh environmental conditions followed the human migration to these areas. There exist a mild directional selective pressure and cross breeding between both breeds; most expected have formed the current genetic diversity status of these breeds. The highest values of F_{IS} were seen for Sahiwal, Dhani, Bhagnari and Dajal. This result could be result of non-random matings within population and might reflects the further intense reproductive management and finally the use of relatively small number of high value bulls as semen donars in assisted reproduction practices. Home tract of these breeds are also far away from each other.

In addition, the partitioning of genetic variation from AMOVA also showed that the foremost amount of genetic variation was always found among individuals within breeds, revealed that 14.6 % of genetic variation was accounted for by differences among populations and 85.4 % was within populations. Significant genetic differentiation was observed among all eleven breeds estimated by F_{ST}= 0.14046. The estimated F_{ST}value in eleven Pakistani cattle breeds was higher than Portuguese taurine local breeds (0.089) (Mateus *et al.,* 2004), Iberian and French breeds (0.07) (Canon *et al.,* 2001; Jordana *et al.,* 2003), Europian taurine breeds (0.112) (MacHugh *et al.,* 1998), Nouthern European breeds (0.107) (Katanen *et al.,* 2000), Zebuine African cattle breeds (0.06) (Ibeagha-

Awemu *et al.*, 2005) and Brazilian Creole cattle breeds (0.098) (Egito *et al.*, 2007). It could be interesting that the overall value estimated of F_{ST} for 21 microsatellite markers used for eleven breeds were significantly high and are useful indicators of markers that could be powerful tools for genetic differentiation of different breeds.

The results of STRUCTURE as reported in this study are indicative that there are subclusters and admixtures between the cattle populations. There appear to be some substantial gene flow between the breeds. Those concerned with the development of breeding plans may want to consider keeping the breed s separate to maintain the genetic identities and diversity between the cattle breeds of Pakistan.

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Statement of conflict of interest

Authors have declared no conflict of interest.

Supplementary Table I is available at http://www.zsp.com.pk/pdf48/QPJZ-0052-2015-F%2021-6-16%20(SUPPLEMENTARY%20TABLES).pdf

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