Advances in Zoology and Botany 3(1): 1-5, 2015
DOI: 10.13189/azb.2015.030101 http://www.hrpub.org

Molecular Genetic Variations among Some Punjab Sheep Breeds Using RAPD Analysis

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Abstract The present study was carried out on seven sheep breeds (*Thalli, Lohi, Salt Range, Kajli, Sipli, Buchi* and *Hissradale*) by Random Amplified Polymorphic DNA (RAPD) analysis using 21 random decamer primers. Blood samples were collected from different numbers of animals per breed, of both sexes. After DNA extraction PCR was carried out by using 25 µL reaction mixture containing 3 m*M* $MgCl_2$, 100 µ*M* each of dATP, dCTP, dGTP, dTTP, 0.2 µ*M* primer, 15 ng of genomic DNA, and 5 unit of *Taq* polymerase, through programmable thermal cycler (Crea Con, TCY, USA).Out of 21 decamer primers 16 primers yielded easily scorable bright DNA bands while other five yielded smeared and nonspecific fragments. The genetic similarities of seven sheep breeds were high, ranging from 74.42% to 94.29 %. *Thalli* and *Latti* had greatest similarity (94.29%). The lowest genetic similarity was between *Lohi* and *Hissardale* (74.42%). The unweighted pair group method of arithmetic means (UPGMA) dendrogram obtained from the cluster analysis revealed two clusters. The study confirms that the molecular genetic techniques such as RAPD-PCR can economically and efficiently be used to establish genetic distances and similarities among and within breeds as well as to find out breed specific genetic markers. The seven sheep breeds in the study could be identified by using one breed specific RAPD marker or a combination of two or more markers. Primer GLB-08 can identify Lohi breed and primer GLA-14 can identify *Hissardale.* Primer GLA-19 can identify both *Kajli* and *Sipli* breeds simultaneously.

Keywords Sheep, Punjab, Thalli, Lohi, Kajli, RAPD-PCR

1. Introduction

Sheep and goat were perhaps the first ruminants to be domesticated around 10,000 B.C. Sheep were first domesticated probably in Iran and Baluchistan and what is now the Near East. Domesticated sheep existed in Harappa and Mohenjo-Daro. Most of the present breeds of sheep have traces of Mediterranean as well as Asian wild sheep [12]. The Pakistani sheep breeds most probably descended from urial (*Ovis vignei*), the wild sheep of Baluchistan, Afghanistan, and the central Asian area, as well as from the argali (*Ovis ammon*), and the Marco Polo sheep of China. At present there are 28 sheep breeds in Pakistan [21]. There are 25.5 million heads of sheep in Pakistan producing 40.7 metric tons of wool and contributing a reasonable amount of mutton (782.1 metric tons) together with goat population [3].

Livestock diversity is shrinking rapidly. Among the domesticated animal populations, it is estimated that 1 to 2 breeds are lost every week. So there is an urgent need to define strategies to prioritize breed conservation [10].The need for conservation comes from the potential rate of decrease of genetic variation. The loss of genetic variation within and between breeds is detrimental. Once animal genetic diversity has been lost, it can not be replaced. Indigenous and locally developed sheep breeds are an important asset for many reasons, particularly because, over time, they have developed unique combinations of adaptive traits to best respond to pressures of local environment [4].

Conservation of genetic resources requires the characterization of the available stock for preservation and management as well as evaluation of the phylogenetic origins of the genetic groups available [18]. In the recent years, several new and sophisticated methods have been developed to screen the polymorphism at the DNA level. A recent technique capable of detecting DNA based polymorphism is random amplified polymorphic DNA (RAPD) analysis. It is based on random amplification using polymerase chain reaction (PCR) with oligonucleotide primers. Single primer can be used to amplify genomic DNA and that polymorphism can be detected between the amplification products of different individuals. Polymorphism between individuals can arise through (1) nucleotide changes that prevent amplification by introducing

a mismatch at one priming site (2) deletion of a priming site (3) insertion that render priming sites to distant to support amplification (4) insertions or deletions that change the size of the amplified product [26].

Efficient use of RAPD markers requires DNA isolation, optimum amplification conditions and appropriate data analysis. Thus, the present study was planned to determine molecular genetic variation among sheep breeds (*Buchi, Hissardale, Kajli, Lohi, Sipli, Thalli and Salt Range*) of Punjab province, Pakistan by using RAPD analysis with the following objectives:

- To optimize conditions for RAPD-PCR amplification for sheep genomic DNA.
- To construct a dendrogram among seven sheep breeds of the Punjab province in Pakistan on the basis of genetic variation.

2. Materials and Methods

The animals from sheep breeds (*Buchi, Hissardale, Kajli, Lohi, Sipli, Thalli and Salt Range*) of Punjab province were sampled according to their spatial distribution keeping in view the breed characteristics and where possible, pedigrees were consulted to ensure that animals were unrelated. Blood samples were collected from animals kept at different livestock farms, under the supervision of Livestock and Dairy Development Department (L&DD) Punjab. Blood samples were taken from various numbers of animals per breed, aseptically from jugular vein in properly labeled 5ml potassium Ethylene Diamine Tetra acetic Acid (EDTA) vacutainers. Blood samples were immediately transferred to ice and later stored at -20^{o}C till further processing. High molecular weight genomic DNA were extracted from the frozen peripheral whole blood according to the FBI protocol reported by Signer *et al.* (1988) and Grimberg *et al.* (1989) with some slight modifications [9,22]. The concentration of total genomic DNA was calculated by measuring Optical Density (OD) at 260 nm (1 OD_{260} = 50 µg of double stranded DNA/ml) on UV visible spectrophotometer.

Table 1. PCR reaction components along with their concentrations

Reagents	1 Rx (µL)
d_3H_2O	7.0 µl
10X PCR buffer	2.5 µl
Gelatin (0.025%)	2.5 µl
$MgCl_2$ (3 m*M*)	3.0 µl
dNTPs (2.5 m*M*)	4.0 µl
Primer (15 ng/µL)	2.0 µl
Taq Polymerase (5 U/µL)	1.0 µl
Template DNA (15 ng/µL)	3.0 µl
Total Volume	25 µL

The DNA bulks of each breed were prepared by pooling equal quantities of DNA from available number of individuals. PCR was carried out in 25µL reaction mixture containing 3 m*M* $MgCl_2$, 100 µ*M* each of dATP, dCTP, dGTP, dTTP, 0.2 µ*M* primer, 15 ng of genomic DNA, and 5 unit of *Taq* polymerase, through programmable thermal cycler (Crea Con, TCY, USA). A total of 21 primers were used for the analysis. The thermal cycler was programmed for 5 minutes initial denaturation at 94^{o}C for first cycle, followed by 1 minute denaturation at 94^{o}C, 1 minute primer annealing at 36^{o}C and 2 minutes extension at 72^{o}C for next 40 cycles and then final extension at 72^{o}C for 10 minutes. PCR reaction components are given under in table 1.

The PCR products were electrophoresed at 50 V in 1.2 % agarose gel for approximately 2 hours using 0.5 X Tris Boric Acid EDTA (TBE) buffer containing ethidium bromide (10 ng/100 mL agarose solution in TBE). The fingerprints were examined under ultra violet illuminator and photographed using the Uvitec Gel Documentation Still Video System.

Amplification profiles of seven sheep breeds were compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The data of the primers were used to estimate genetic similarity (GS) on the basis of number of shared amplification products [16]. The coefficients were calculated by the following statistical equation;

$$GS_{(ab)} = 2N_{ab} / (N_a + N_b)$$

$$D_{(ab)} = -\ln (GS_{ab})$$

Where,

GS is the genetic similarity coefficient and D is the genetic distance in which N_a and N_b are the numbers of fragments in breed *a* and *b*, respectively, whereas Nab is the fragment shared by the two populations.

Similarity coefficients were utilized to generate a phylogenetic tree (dendrogram) by using unweighted pair group method of arithmetic means (UPGMA) [23].

3. Results and Discussion

Pooled DNA of seven sheep breeds were amplified with 21 different random primers. Five primers amplified no fragments or yielded smeared bands that could not be clearly identified. A total of 53 DNA fragments were generated by the remaining 16 primers with an average of about 3.3 bands per primer. The number of DNA fragments amplified with each primer was in a range reported in different studies using the random primers [5,19]. Bands that a primer yielded in this study ranged from one to six. Generally, the size and number of the fragments produced strictly depended upon the nucleotide sequence of the primer used and upon the source of the template DNA. To check the consistency of the amplified products, reactions were duplicated from time to time. Only easily resolved bright DNA bands were considered and scored.

All the breeds showed variation with each other on their amplification profile bases. Of the total 53 DNA bands amplified by 16 primers, 16 fragments showed polymorphism among seven sheep breeds, which is an approximately 30.19% polymorphism. The rest of the 37

bands (69.81%) were monomorphic in all seven sheep breeds. These results indicated that the level of DNA variation was low among the seven sheep breeds. These seven sheep breeds could be identified by using one breed specific RAPD marker or a combination of two or more markers. Primer GLB-08 can identify *Lohi* breed and primer GLA-14 can identify *Hissardale.* Primer GLA-19 can identify both *Kajli* and *Sipli* breeds simultaneously. Therefore RAPD markers can be used for identification of sheep breeds. Out of seven breeds studied, *Kajli* sheep produced the maximum number of DNA fragments (46) while minimum numbers of fragments (40) were produced by *Hissardale*.

Table 2. List of sixteen RAPD primers amplified and total number of fragments scored for each primer

Primer	Amplified Fragments	Primer	Amplified Fragments
GLA-08	1	GLB-04	6
GLA-13	3	GLB-05	1
GLA-14	3	GLB-06	4
GLA-15	2	GLB-07	4
GLA-18	3	GLB-08	4
GLA-19	2	GLB-10	5
GLA-20	3	GLB-15	4
GLB-01	4	GLB-17	4

Genetic Similarity among sheep breeds

The genetic similarity matrix of RAPD data for the seven sheep breeds was constructed based on Nei and Li's (1979) coefficient of similarity and is shown in table. The genetic similarity of seven sheep breeds were high, ranging from 0.9429 to 0.7442.

Genetic Similarity among sheep breeds

Nei's standard genetic distance (D) was calculated from RAPD-PCR data to estimate the molecular genetic variation among seven sheep breeds. Genetic distance among these breeds was very low in the range of 0.0588 to 0.2954. the maximum genetic distance was found between *Lohi* and *Hissardale,* while, minimum genetic distance was found between *Thalli* and *Salt Range (Latti)* (0.0588).

Template DNA concentration of 15 ng/uL was found to be optimum. The optimum concentration of $MgCl_2$ was found to be 3 m*M*. $MgCl_2$ concentration has a profound effect on DNA amplification. Five unit/25uL reaction mixtures were found optimum. The optimum annealing temperature found in this study was 36^{o}C. Forty cycles per reaction was found optimum. Random amplified polymorphic DNA (RAPD) produced consistent results with optimized conditions and have potential to be employed for molecular genetic variations and taxonomy classification. The observed polymorphism in the present study was 30.19%, while 69.81% fragments were monomorphic. The probability of finding these short decamer primer cognate sequences in the target DNA varies and may be numerous, such that a single primer generates multiple amplification fragments.

Forty two animals were analyzed out of the sixty nine selected for blood collection. Genomic DNA of six animals from each breed was extracted and pooled to make bulks by mixing equal quantity of DNA from each individual. Nei's standard genetic distance among seven breeds was very low in the range of 0.2954 to 0.0588. In the present study the maximum genetic distance was found between *Lohi* and *Hissardale* (0.2954), while, minimum genetic distance was found between *Thalli* and *Salt Range (Latti)* (0.0588). On the other hand the genetic similarities of seven sheep breeds were high, ranging from 94.29 % to 74.42 %. *Thalli* and *Latti* had greatest similarity (94.29 %). The unweighted pair group method of arithmetic means (UPGMA) dendrogram obtained from the cluster analysis revealed two clusters.

Table 3. Similarity Matrix of seven sheep breeds obtained from RAPD markers

Breeds	Thalli	Lohi	Salt Range	Kajli	Sipli	Buchi	Hissardale
Thalli	1						
Lohi	0.9102	1					
Salt Range	0.9429	0.8471	1				
Kajli	0.8414	0.8033	0.8428	1			
Sipli	0.9020	0.8639	0.9034	0.9380	1		
Buchi	0.9388	0.8490	0.8830	0.8525	0.9224	1	
Hissardale	0.7823	0.7442	0.8346	0.8653	0.8442	0.7666	1

Table 4. Nei's standard genetic distance (D) among seven sheep breeds of the Punjab province.

Breed	*Thalli*	*Lohi*	*Salt Range*	*Kajli*	*Sipli*	*Buchi*	*Hissardale*
Thalli	0	0.0941	0.0588	0.1727	0.1031	0.0632	0.2455
Lohi		0	0.1659	0.2190	0.1463	0.1637	0.2954
Salt Range			0	0.1710	0.1016	0.1244	0.1808
Kajli				0	0.0640	0.1596	0.1447
Sipli					0	0.0808	0.1694
Buchi						0	0.2658
Hissardale							0

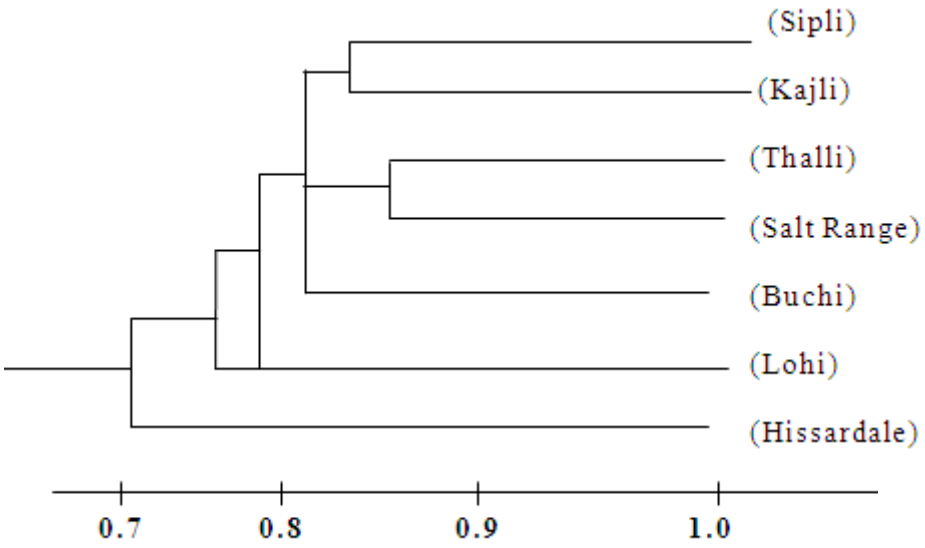

Figure 1. Phylogenetic relationships among some Punjab Sheep breeds generated by RAPD data using UPGMA method

4. Conclusions

Genetic distance among these breeds was low in the range of 0.0588 to 0.2954. The low levels of genetic variation among these breeds indicate the high degree of genetic relationships. This increase in the relatedness among these indigenous breeds is the result of haphazard and indiscriminate crossbreeding in the home tracts of the respective breeds, and lack of the implementation of the provincial and national breeding policies in the country. For molecular genetic studies, sophisticated inputs such as; authenticity of pedigree, appropriate selection of molecular technique, availability of a prior knowledge about the genome of respective species, stipulation of good working conditions, latest equipments and laboratories are of supreme importance. A large number of primers from different companies, more number of animals from each breed with authentic pedigree recording and sampling from breed home tracts is also suggested along with more advanced molecular genetics techniques using microsatellites such as SSR, in order to refine the relationship.

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