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MOLECULAR CHARACTERIZATION OF SELECTED LOCAL CATTLE USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

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Abstract

A genetic analysis using RAPD (Random Amplified Polymorphic DNA) markers was performed to determine the cattle-specific primers and generate RAPD fingerprints to find out genetic diversities of cattle breeds an important province for cattle. The DNA samples were isolated from a total of twenty animals from local cattle and three random primers were selected. Genetic relations among cattle were determined by RAPD polymorphisms. The study showed that the polymorphisms generated by RAPD-PCR enabled the determination of genetic relationships and fingerprints of local cattle. In this study, molecular characteristics were used to determine genetic relationship among these selected local cattle. The results obtained from amplified PCR products are more reliable than that of morphological characters. In this experiment the highest value of Nei's genetic distance was found among Bag-03, Pir-85, Pir-10, Pir-01, Khu-09, and Khu-11and Khu-17 cattle. The lowest genetic distance was revealed between Bag-102 and Bag-69 cattle. A total of 14 bands were identified from PCR products which illustrated 86.66% of the polymorphism. These data provides a scientific basis for future studies on local cattle.

Keywords: Cattle breeds, genetic diversities, cattle-specific primers, RAPD fingerprints

Introduction

Bangladesh is known as an agricultural country. Livestock, being one of the major components of agricultural output (crops, livestock, fisheries and forestry) plays a vital role in national economy, contributing about 6.5% of Gross Domestic Product (GDP) and 13% of total foreign exchange earning (DLS, 2008). The total ruminant livestock population of Bangladeshis composed of 24.0 million cattle, 34.4 million goats, 0.83 million buffalos and 1.14 million sheep (Omore *et al.,* 2002). Dairying is considered a strong tool to develop a village micro economy of Bangladesh (Shamsuddin *et al.,* 2006) in order to improve rural livelihoods and to alleviate rural poverty. Domesticated animals, especially livestock and poultry, are an important source of protein in Bangladesh. Increasing this protein resource requires the conservation of diversity among indigenous livestock. The wide range of breeds and species that have evolved in various environments represent unique sets of genetic diversity. It has been estimated that since domestication, over 6,379 documented breed populations from 30 species of livestock have been developed globally in the last 12 thousand years (FAO, 2000). Recently, loss of genetic diversity within indigenous livestock breeds has been a major concern. The primary aim of studying genetic diversity is to understand the extent of differentiation of populations within species. Population-specific genetic markers (alleles) can be generated using a range of methods available for detection of polymorphic loci. Polymorphic genetic markers are extremely useful for a number of applications, such as measurement of the amount of genetic diversity in species, discrimination between individuals, strains or species, identification of markers linked to economically useful traits as well as analyses of animal kinship relationships, behavioral and population ecology. Now, this molecular approach became a powerful and reproducible tool in genetic analysis, breeding and identification of species and/or populations. In the case of cattle, lots of researchers have been made to detect DNA polymorphisms between cattle breeds by RAPD analysis during last decades. Especially genetic markers for native cattle's in several countries were investigated for the improvement of their native cattle, including Japanese Black cattle (Wagyu), German native cattle, Zebu cattle, *Bos indicus*, and *Bos Taurus.* Considering the above statement, the present research work on molecular characteristics of selective local cattle using random amplified polymorphic DNA (RAPD) markers was undertaken to know the genetic diversity and relationships among local cattle.

Materials and methods

The experiment was carried out at the Animal cell culture and Molecular Laboratory in Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh. The details of materials and methods used are described as follows.

Animal selection and blood sample collection

In order to minimize sampling errors two conditions were maintained while selecting local cattle:

- Related animals were not sampled.
- Sampling of animals which represented different eco-regional populations.

Bagerhat, Khulna and Pirojpur districts were considered suitable for these conditions because animals from different regions were frequently introduced and maintained in semi-intensive farming system. A total of twenty native cattle were selected for this study. Five ml blood was collected from each animal in a 10 ml EDTA vaccutainer tubeand kept in ice box during collection and then transported to the laboratory and stored at -20ºC until the isolation of genomic DNA. The cattle's identity no., source and sex are given in Table 1.

Table 1. Cattle's identity numbers, sources and sexes

SL. No.	Cattle No.	Source	Identity	Sex
1	Bag-27	Kachua, Bagerhat.	Local	Male
2	Bag-99	Kachua, Bagerhat	Local	Male
3	Bag-102	Kachua, Bagerhat	Local	Female
4	Bag-50	Kachua, Bagerhat	Local	Female
5	Bag-69	Kachua, Bagerhat	Local	Female
6	Bag-03	Kachua, Bagerhat	Local	Male
7	Bag-35	Kachua, Bagerhat	Local	Male
8	Bag-05	Kachua, Bagerhat	Local	Female
9	Pir-85	Nazirpur, Pirojpur	Local	Female
10	Pir-05	Nazirpur, Pirojpur	Local	Female
11	Pir-07	Nazirpur, Pirojpur	Local	Male
12	Pir-10	Nazirpur, Pirojpur	Local	Female
13	Pir-01	Nazirpur, Pirojpur	Local	Female
14	Pir-13	Nazirpur, Pirojpur	Local	Female
15	Khu-09	Pabla, Khulna.	Local	Male
16	Khu-02	Pabla, Khulna.	Local	Female
17	Khu-03	Pabla, Khulna.	Local	Male
18	Khu-11	Pabla, Khulna.	Local	Male
19	Khu-12	Pabla, Khulna.	Local	Female
20	Khu-17	Pabla, Khulna.	Local	Female

Extraction of genomic DNA from blood sample

DNA was extracted from blood by following the modified method of Bruce (1996). The presence of DNA in the extracted samples was confirmed by running the sample in a 1 % agarose gel at 50V for 1.20 hours. The quantity of DNA was measured by spectrophotometer at 260 nm wavelength.

PCR reaction

Primer selection

In this study 3 RAPD primers were selected after screening 10 primers (Bioneer). List of the primers along with their sequences are shown in Table 2.

Table 2. List and sequences of primers used in the experiment

Primer No.	Sequence (5'-3')	(G+C) Contents %
01	AATGCTGG	40%
02	TCAGCCACGT	60%
03	TGCCTCGCACCA	80%
04	TCTGCCATCG	60%
05	CTTATCAGGG	50%
06	TTCCCCCCAG	70%
07	GGTGACGCAG	70%
08	GGCCCTACAT	60%
09	GTTGCCACCC	70%
10	AACGGTGACC	60%

PCR amplification

The amplification conditions were based on Williams *et al.* (1990). PCR reactions were performed on each DNA sample in a 20 µl reaction mix containing 2 µl of template (4ng DNA), 2 µl of 10x reaction buffer with $MgCl_2$, 2 µl of dNTPs (250 mM each), 2 units of Taq DNA polymerase, 1 µl (100 picomol) of primer, 11 µl of sterile distilled water. DNA amplification was performed by thermal cycler (Eppendrof Master Cycler Gradient). The amplification program included an initial denaturation step of 94°C for 5 minutes (one cycle) followed by 40 cycles of 94°C (denaturation), 1 minute; 37°C (annealing), 1 minute and 72°C (extention) for 2 minutes. The final extension was performed at 72°C for 15 min (one cycle) and held at 8°C.

Gel electrophoresis of PCR amplified Product

PCR products from each sample were confirmed by running 2 % agarose gel in 1x TAE buffer at 40V for 80 minutes. Loading dye (5 µl) was added to the PCR products and loaded in the wells. Two molecular weight markers DNA (ladder) were also loaded on one side of the gel. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber, washed with 7 µl ethidium bromide, mixed with distilled water (500 ml) and placed on high performance ultraviolet light box (UV transilluminator) for checking the DNA bands and photographed using a digital camera.

Data analysis

Although a large number of fragments were generated from each primer, only clearly distinguishable and

reproducible bands were considered and data was entered in a computer file as a binary matrix "0" coded for absence and "1" for presence of a band. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This was used for estimating polymorphic loci, Nei's (1972) gene diversity, gene frequency, genetic distance (COMPONENT ,Version 1.31) (Yeh *et al.,* 1999). Dendrogram was constructed using Unweighted Pair Group Method using Arithmetic Averages (UPGMA).

Results and Discussion

Data regarding molecular characteristics were used to evaluate different levels of variability among experimental local cattle. Molecular characterization was determined using RAPD markers.

DNA extraction and Quantity determination

The spectrophotometer absorbance readings at 260 nm of twenty experimental local cattle shown in Table 3.

Table 3. Spectrophotometric absorbance reading at 260 nm of twenty experimental local cattle for DNA quantification

Sl. No.	Accession or tag no.	Absorbance reading at 260 nm	Quantity of DNA(ng/ul)
1	ag-27	0.049	245
2	Bag-99	0.043	215
3	Bag-102	0.025	125
4	Bag-50	0.038	190
5	Bag-69	0.044	220
6	Bag-03	0.039	195
7	Bag-35	0.030	150
8	Bag-05	**0.063**	**315**
9	Pir-85	**0.023**	**115**
10	Pir-05	0.040	200
11	Pir-07	0.032	160
12	Pir-10	0.053	265
13	Pir-01	0.056	280
14	Pir-13	0.028	140
15	Khu-09	0.037	185
16	Khu-02	0.042	210
17	Khu-03	0.035	175
18	Khu-11	0.028	140
19	Khu-12	0.039	195
20	Khu-17	0.041	205

Molecular characterization

In the present study, a polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) method was used to amplify local cattle DNA using arbitrary oligonucleotide primers. The method was able to detect the heterogeneity of amplified DNA from the breeds of cattle. The present study indicates the effectiveness of RAPD analysis in detecting the level of polymorphisms among the experimental local cattle. Three effective primers were selected for determination the polymorphism of twenty genotypes.

Primer Selection and RAPD Analysis

All the ten primers used in the RAPD analysis were 10-12 bps long with GC content of 60-80%. Primers with ten nucleotides and a (G+C) content of at least 50% are generally employed, because primers having high (A+T) content may cause DNA-primer hybrid melting during polymerization at 73^0 C (Weising *et al.*, 1995). Three selected primers used in the RAPD analysis of twenty experimental cattle amplified twenty different reproducible bands.

Different RAPD patterns generated by the primers are represented below.

From the present study primer OPA-03 and OPA-05 were found to be more reproducible and produced more distinct RAPD profile than the primer OPA-02. These three primers amplified 14 different reproducible bands. Three different primers generated various banding patterns and of the 14 bands scored, 12 bands (86.66%) were found to be polymorphic and 2 bands (40%) were found to be monomorphic in nature. The frequencies of polymorphic bands varied from primer to primer. Though no experimental local cattle-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different experimental cattle. Experimental cattle could be distinguished by a combination of fragments and differences between clusters reflected differences in frequencies rather than presence or absence of experimental local cattle specific fragments. The amplification patterns of representative samples of cattle with three different primers have been shown in Fig 1, Fig 2 and Fig 3. The frequencies of polymorphic bands varied from primer to primer. Though no experimental local cattle-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (86.66%) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different experimental local cattle. Experimental local cattle could be distinguished by a combination of fragments and differences between clusters reflected differences in frequencies rather than presence or absence of experimental local cattle specific fragments.

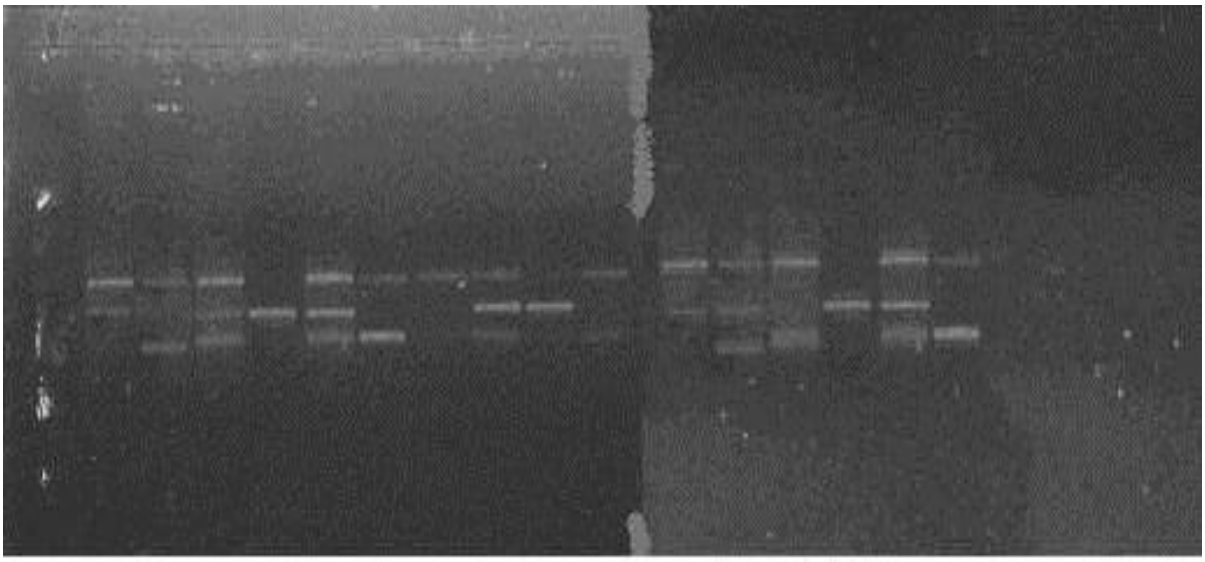

Fig 1. RAPD pattern generated using primer-5 of twenty experimental local cattle. The number corresponds to the serial number of the genotypes

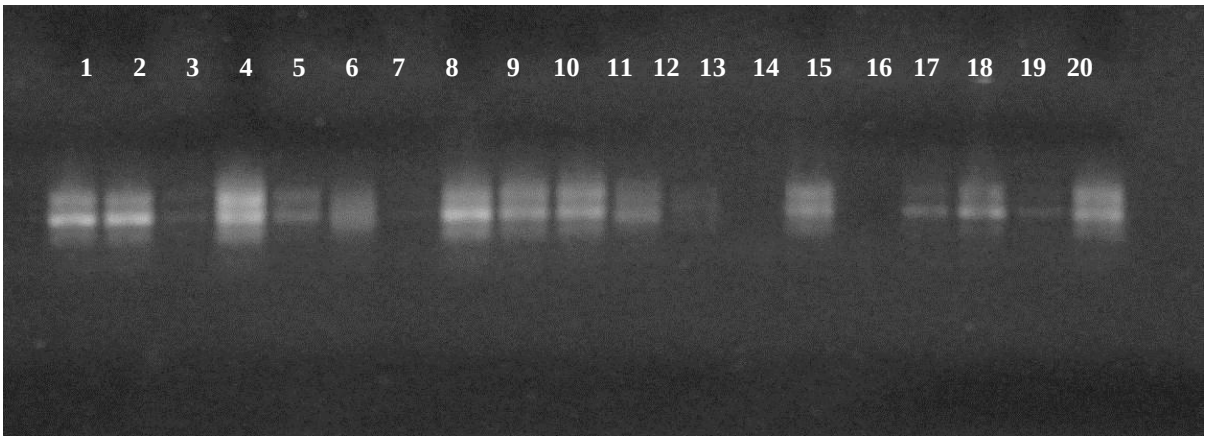

Fig 2. RAPD pattern generated using primer-3 of twenty experimental local cattle. The number corresponds to the serial number of the genotypes

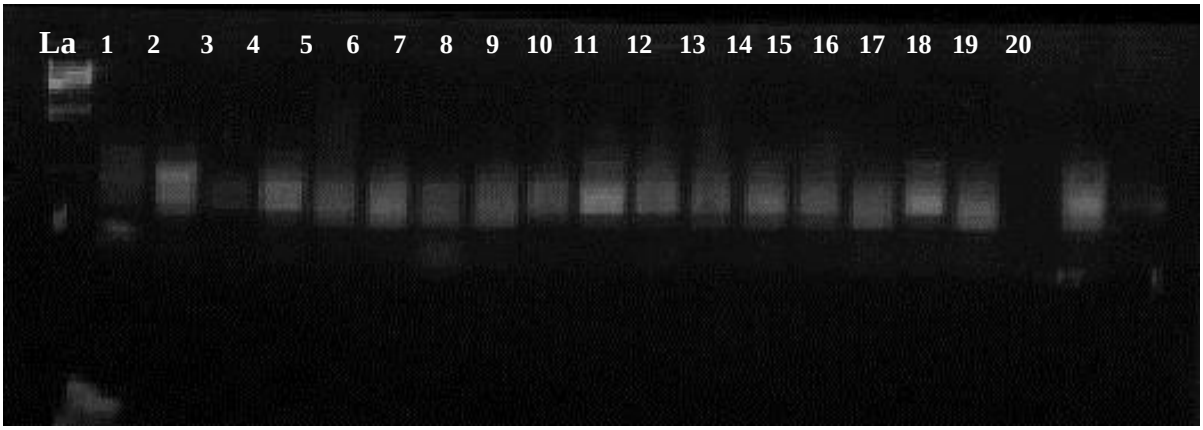

Fig 3. RAPD pattern generated using primer-2 of twenty experimental local cattle. The number corresponds to the serial number of the genotypes

Table 4. List of primers and their monomorphic and polymorphic bands

Serial No.	Primer code	No. of Total bands	No. of Monomorphic bands	No. of Polymorphic bands	Polymorphic Loci (%)
1	OPA-5	4	0	4	100
2	OPA-3	5	2	3	60
3	OPA-2	5	0	5	100
	Total 3	14	2	12	Av. =86.66

Genetic Distance

Estimates of (Nei's and Li 1979) genetic distance calculated all possible pairs-wise genetic distance values by the following formula: $d_{xy} = 1-\{2n_{xy}/(n_x + n_y)\}$

Where, n_x and n_y are the numbers of bands amplified in individuals x and y, respectively, and $2n_{xy}$ is he number of bands shared by those individuals. In this analysis, smaller numbers are associated with more genetically similar individuals, whereas larger numbers suggest genetically dissimilarity.

In the present experiment, comparatively higher genetic distances (0.90) were found among Pir-10 and Khu-11, Pir-01 and Khu-11, Khu-09 and Khu-11 cattle. The lowest genetic distance (0.05) was observed between Bag-102 and Bag-69, Bag-99 and Bag-69 cattle. The range of genetic distance of twenty genotypes was 0.05-0.90. The difference between the highest and lowest genetic distance indicated the presence of variability among twenty genotype local cattle. The genetic diversity of Pirojpur and Khulna was relatively higher for prescribed local cattle. The results revealed that there was a high level (86.66%) of genetic variation among the experimental cattle as indicated by the proportion of polymorphic loci. Estimation of higher level of genetic variation in the local cattle might be consistent with the fact that it is a highly polymorphic animal. The values of pair-wise genetic distance among twenty experimental cattle genotypes were computed from combined data sets for three primers ranging from 0.05 to 0.90 (Table 5). Total 14 bands were identified from PCR products which illustrated 86.66**%** polymorphism. The higher degree of polymorphism was 86.66**%** compared to 28 % in taurine cattle (Talle *et al*., 2005) and 18 % in zebu cattle (Marle-Koster *et al*., 2003).

In one study involving indigenous African cattle, Gwakisa *et al.* (1994) used RAPD markers to characterize the local Zebu (*Bos indicus*) cattle breeds of Tanzania. Using RAPD markers, the relatedness among the three local breeds of Tanzania was quantified. One of the primers, ILO 1127, amplified a RAPD fragment in 61% of the Tanganyika Shorthorned Zebu animals but less than 6% in the other breeds. Another primer, ILO 1065, revealed a DNA segment common to 89% of the Boran animals and less than 30% in the other two breeds evaluated. Further, the study revealed that ILO 1065 primer could be a *Bos indicus*-specific Y-linked polymorphism. They also showed that RAPD analysis could detect introgression.

In one of the few studies reported, Mwacharo *et al.* (2006) phenotypically characterized two breeds of Zebu in Kenya. They used a total of 12 morphometric measurements to show that the Masaai Zebu was different from the Kamba Zebu in Kenya. The apparent wide within-breed and between-breed variations in linear body dimensions that were observed in the study were indicative of the large genetic diversity inherent in the small East African Zebu cattle, with clearly well-differentiated breeds. The advent of molecular techniques has led to an increase in the studies that focus on the genetic characterization of domestic breeds using genetic markers (Giovambattista *et al*., 2001).

Cluster Analysis of the Genotypes Based On RAPD Analysis

Genetic similarities measured through analysis of data using three RAPD markers from the twenty genotypes of local cattle revealed varying degrees of genetic relatedness. According to Nei and Li (1979) the genetic distance from 0 to 1. The highest dissimilarity coefficient (0.90) was observed among genotypes of local cattle. Using the Nei's (1979) genetic distance, a dendrogram was constructed to obtain the clustering of local cattle. The dendrogram shows that all the cattle were found to be grouped in two major groups designated as I, II, IIA, IIB, IIA(1), IIA(2), IIB(1) and IIB(2) . The distribution of the cluster members is shown in (Table 6). Cluster II is broad which includes seventeen local cattle. Cluster I includes the highest genetic distance. Cluster II divided into two sub-cluster (IIA & II B) & II a showed the less variation and cluster forms separately but II B again divided into sub-cluster and that cluster shows the highest genetic similarity because of their lowest (0.05) genetic distance value.

Construction of phylogenic tree

The UPGMA dendogram based on the Nei's genetic distance differentiated the varieties into two main clusters-I and II. Cluster II is further divided into two sub-cluster, IIA and IIB which were further subdivided into II A(1), II A(2) and II B(1), II B(2) respectively.

Table 5. Estimation of pair wise genetic distance between experimental local cattle using Nei's equation

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	****																			
2	0.33	***																		
3	0.23	0.05	***																	
4	0.14	0.50	0.263	***																
5	0.23	0.05	0.05	0.23	***															
6	0.454	0.38	0.33	0.45	0.33	***														
7	0.40	0.20	0.27	0.80	0.27	0.33	***													
8	0.33	0.50	0.07	0.33	0.07	0.45	0.40	***												
9	0.25	0.40	0.27	0.80	0.27	0.55	0.50	0.40	***											
10	0.23	0.20	0.14	0.23	0.14	0.16	0.27	0.384	0.454	***										
11	0.50	0.33	0.23	0.33	0.23	0.09	0.17	0.33	0.60	0.230	****									
12	0.80	0.40	0.27	0.20	0.27	0.33	0.50	0.40	0.90	0.636	0.40	***								
13	0.45	0.45	0.50	0.45	0.50	0.20	0.33	0.636	0.77	0.50	0.454	0.333	***							
14	0.23	0.23	0.57	0.07	0.14	0.33	0.45	0.230	0.272	0.285	0.538	0.636	0.50	***						
15	0.50	0.33	0.23	0.50	0.23	0.27	0.40	0.333	0.60	0.538	0.33	0.20	0.272	0.384	***					
16	0.23	0.23	0.28	0.23	0.28	0.33	0.27	0.384	0.454	0.285	0.23	0.454	0.166	0.285	0.384	***				
17	0.55	0.33	0.40	0.33	0.40	0.75	0.42	0.333	0.428	0.60	0.55	0.714	0.75	0.40	0.77	0.40	***			
18	0.66	0.71	0.75	0.28	0.75	0.55	0.60	0.714	0.60	0.75	0.714	0.90	0.90	0.75	0.90	0.75	0.50	***		
19	0.40	0.20	0.72	0.20	0.27	0.44	0.75	0.60	0.25	0.454	0.40	0.75	0.555	0.272	0.60	0.272	0.142	0.60	***	
20	0.75	0.50	0.50	0.66	0.55	0.42	0.33	0.75	0.66	0.777	0.75	0.66	0.428	0.555	0.50	0.555	0.60	0.90	0.333	***

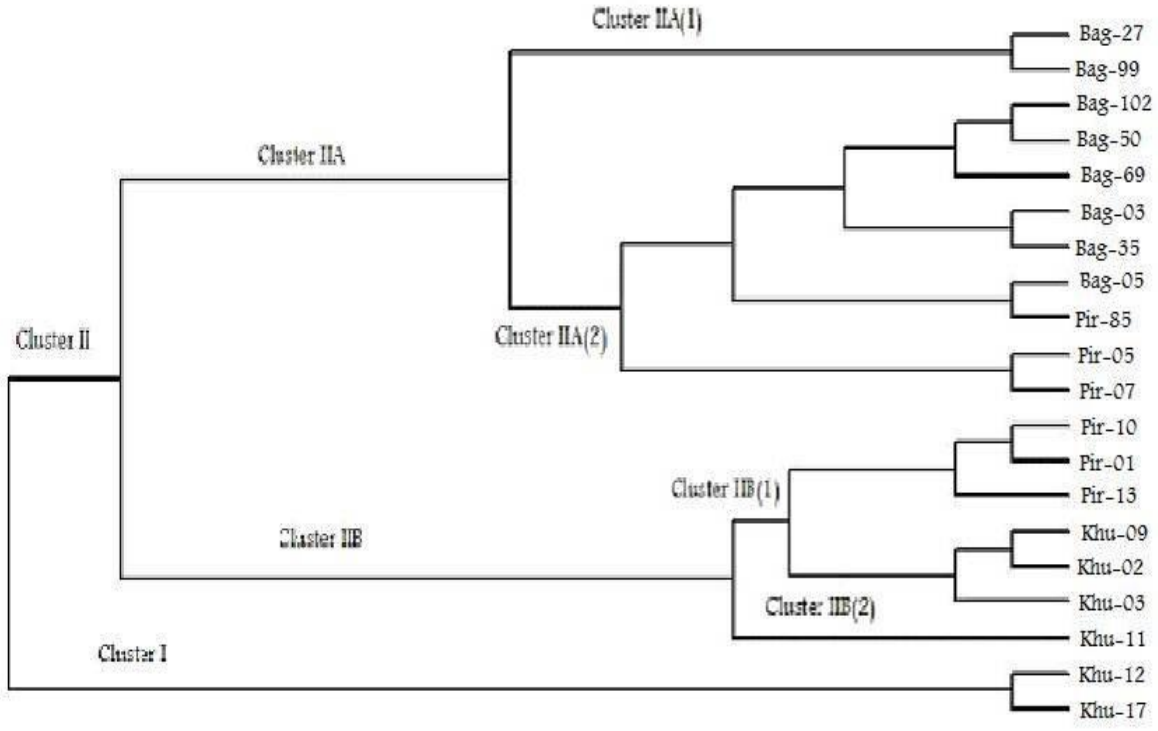

Fig 4. Unweighted pair group method with arithmetic mean (UPGMA) dendogram based on summarized data regarding differentiation among experimental local cattle

Table 6. Distribution of twenty experimental cattle under different cluster

Cluster no			Total no. of cattle in cluster	cattle included in different clusters
I			3	Khu-11, Khu-12, Kku-17
II	II A	II A(1)	9	Bag-27, Bag-99, Bag-102, Bag-50, Bag-69, Bag-03, Bag-35,Bag,-05, Pir-85
		II A(2)	1	Pir-07
	IIB	II B(1)	5	Pir-10,Pir-01, Pir-13, Khu-09, Khu-02
		II B(2)	1	Khu-03

Discussions

Genetic diversity is of great significance for breeding programmes. Germplasms characterization is an important component of programmes on effective and efficient management/utilization of animal genetic resources. A maximum of 6 polymorphic bands were obtained in PCR amplification from genomic DNA of the cattle used in the present study. RAPD markers are randomly distributed throughout the genome, but most regions of the genome (86.66 %) are not expressed at the phenotypic level. As a result, markers like RAPDs may accurately assay the degree of genetic change between two genomes, but they may not necessarily reflect the divergence in terms of changes in traits of livestock importance.

The differences of genetic variations in the present study found in twenty experimental cattle samples were high and therefore, there is a scope for future improvement of local cattle. More research needs to be done on the native cattle and the choice of cattle for conservation must take into account any available information on productivity traits of economic value, specific adaptive features (tolerance to heat, low quality feeds, disease etc.), presence of unique genes or phenotypes, local or regional importance of a breed in production systems, and the availability of resources and infrastructure for animal production.

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