ISSR markers for assessing DNA polymorphism and genetic characterization of cattle, goat and sheep populations

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Abstract
Based on the purpose of conservation planning for native species, sixteen populations of cattle, goat and sheep were analyzed by amplification of genomic DNA using inter-simple sequence repeat (ISSR) markers to estimate of genetic structure. DNA samples of 275 animals were collected to Paccess their genetic content. The polymorphism information content (PIC) values and genetic diversity in sheep populations were higher than the others. The mean coefficient of gene differentiation (Gst) was 0.3615, indicating that 19.42% of the genetic diversity resided within the population. In total, 60 fragments in PCR products were indicated by using ISSR primers and generally most of the fragments were common in all populations, but differed in their frequency. A cluster analysis was carried out using unweighed pair group method with arithmetic averages (UPGMA) and dendrogram illustrated genetic relationships among 275 individuals in three species. Haplotypes were constructed computationally and frequencies were compared in each species. The results of this study can provide basic molecular information for future research on native livestock using ISSR markers.
***Keywords*:** ISSR markers; native livestock; polymorphism information content; UPGMA dendrogram; haplotype

INTRODUCTION

A species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites (Schaal *et al*., 1991) and also the ability of a population to respond adaptively to environmental changes depends on the level of genetic variability or diversity it contains (Ayala and Kiger, 1984). Therefore, studies of population genetic diversity and the structure of population within and between species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation of the Bovidae family, sheep, goats, birds and so on (Notter, 1999). Maintaining the integrity of livestock species as well as their genetic diversity is one of the paramount interests for agricultural policies and serious steps are necessary to be taken in order to protect the stocks of local animals which are in danger of extinction. The preservation of the biodiversity can be achieved through the protection of genes reserves. One major use of DNA techniques in conservation is to reveal genetic diversity within and between populations inter-simple sequence repeat (ISSR) which is a DNA and can be used without knowing the sequence information for genomic DNA (Zietkiewicz *et al*., 1994). The ISSR marker technique involves polymerase chain reaction (PCR) amplification of DNA using a single primer composed of a microsatellite sequence, The ISSR has mild technical difficulty, good reproducibility and reasonable cost, permitting its use for genetic studies of population (Li and Xia, 2005; Chen *et al*., 2005). Kerman is one of the 30 provinces of Iran. It is located on a high margin of Kavir-e Lut (*Lut Desert*) in the central south of Iran and in its vast climates there are some kinds of local animals. Three kinds of them

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are: Kermani sheep, Rayini Cashmere goat and Local cattle. They have unique characteristics and traits such as resistance to disease or adaptation to climatic extremes that could prove fundamental to the food security of future generations. In this study, we investigated the genetic diversity and population structure of these three species as well as their relationship with the Holstein cattle.

MATERIALS AND METHODS

Animals: Sixteen populations of animals were used in this study. They were obtained from the different regions of Kerman province, containing: four populations of Rayini goat experimental station located near the city of Baft; three populations of Kermani sheep, in which system of production consisted of hill, village and intensive operation in Shahr-e Babak; five populations of local cattle which are well adapted to the local environment of Bam city and four populations of Holstein cattle that occur all around the Kerman city, as a breed of dairy cow known today as the world's highest production dairy animal. Each population consisted of (8-31) animals (Table 1).

Genomic DNA extraction: Blood samples were from 275 animals (containing 145 cattle, 70 goats and 60 sheep) were collected into vacutainers with EDTA as anticoagulant. Samples were kept at -20°C until use. DNA was extracted from the whole blood using an optimized and modified salting-out method (Miller *et al*., 1988).

ISSR amplification: The PCR amplification was performed in a 25 µl reaction volume, containing negative controls, using CinnaGen PCR Master Kit according to the instructions by the manufacturer (CinnaGen Co., Iran). The negative control contained sterile water instead of DNA template. Initial denaturation for 5 min at 94°C was followed by 35 cycles of 1 min at 94°C, 1 min at specific annealing temperature, 2 min at 72°C and a 10 min final extension step at 72°C (Table 1). Amplification products were electrophoresed on 2%

Table 1. List of primers used for ISSR amplification, annealing temperature (Tm).

Primer	Primer Sequence (5'-3')	Annealing Temperature (°C)
P01	5'-AGA GAG AGA GAG AGA GAG C-3'	55
P02	5'-GAG AGA GAG AGA GAG AGA C-3'	55

Table 2. Sixteen populations of animals collected from different sites of province.

Populations	Sample Size	Sampling site	Na*	Ne	H	I	No. of polymorphic loci	Percentage of polymorphic loci (%)
Holstein (1)	9	Kerman	1.3833(0.4903)	1.1399(0.2712)	0.0891(0.1467)	0.1449(0.2167)	23	38.33
Holstein (2)	24	Kerman	1.8167(0.3902)	1.1686(0.2040)	0.1240(0.1204)	0.2204(0.1769)	49	81.67
Holstein (3)	24	Kerman	1.6000(0.4940)	1.1298 (0.2059)	0.0938(0.1202)	0.1661(0.1833)	36	60.00
Holstein (4)	31	Kerman	1.6167(0.4903)	1.1757(0.2247)	0.1234(0.1404)	0.2066(0.2138)	37	61.67
Local cattle (1)	14	Bam	1.3333(0.4754)	1.1532(0.2805)	0.0951(0.1597)	0.1481(0.2359)	20	33.33
Local cattle (2)	15	Bam	1.4667(0.5031)	1.1426(0.2469)	0.0950(0.1425)	0.1576(0.2128)	28	46.67
Local cattle (3)	8	Bam	1.4333(0.4997)	1.2187(0.3040)	0.1367(0.1761)	0.2107(0.2607)	26	43.33
Local cattle (4)	8	Bam	1.3833(0.4903)	1.1679(0.2734)	0.1073(0.1601)	0.1690(0.2389)	23	38.33
Local cattle (5)	12	Bam	1.4833(0.5039)	1.2457(0.3193)	0.1517(0.1826)	0.2328(0.2678)	29	48.33
Goat (1)	20	Baft	1.2667(0.4459)	1.1259(0.2721)	0.0759(0.1526)	0.1169(0.2330)	16	26.67
Goat (2)	15	Baft	1.2667(0.4459)	1.1085(0.2624)	0.0655(0.1410)	0.1034(0.2058)	16	26.67
Goat (3)	15	Baft	1.2333(0.4265)	1.1401(0.2991)	0.0811(0.1634)	0.1211(0.2366)	14	23.33
Goat (4)	20	Baft	1.3333(0.4754)	1.1766(0.3149)	0.1052(0.1726)	0.1604(0.2110)	20	33.33
Sheep (1)	20	Shahr-e Babak	1.5500(0.5017)	1.3576(0.4146)	0.1994(0.2149)	0.2938(0.3016)	33	55.00
Sheep (2)	20	Shahr-e Babak	1.5500(0.5017)	1.3673(0.4050)	0.2076(0.2120)	0.3058(0.3004)	33	55.00
Sheep (3)	20	Shahr-e Babak	1.5667(0.4997)	1.3512(0.4006)	0.1998(0.2087)	0.2969(0.2946)	34	56.67

*Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei's genetic diversity; I = Shannon's Information Index; Mean = Na, Ne, H and I of all over loci of 16 populations; Ht = Total gene diversity.

agarose gels at constant voltage and 1X TBE for approximately 2 h. They were visualized by staining with ethidium bromide and photographed under ultraviolet light and molecular weights were estimated using DNA markers.

Data analysis: ISSR amplified fragments were scored for band presence (1) or absence (0) and a binary qualitative data matrix was constructed. Data analyses were performed using the NTSYS PC version 2.02 computer package program (Rohlf, 1998 a). The similarity values were used to generate a dendrogram via the un-weighted pair group method with arithmetic average (UPGMA) (Rohlf, 1998 b). Measurement of diversity including gene diversity (H), observed number of alleles (Ne), gene flow and Shannon's information index were estimated by POPGEN 3.2 software (Yeh *et al*., 1999) and Polymorphism information content (PIC) value was estimated using HET software (Ott; 1988-2001).

RESULTS

Two ISSR primers were used to screen 275 randomly selected animals from 16 populations. The negative controls had no template DNA and ther was no indication of amplification. If amplification did occur, it was due to contamination in the reagents, thus indicating that the bands in the other samples were likely contaminated as well. A total of 60 ISSR loci were detected. The amplified PCR fragment sizes ranged from 100 to 3100 bp. The range of polymorphic loci was between 26.67%-81.67%. The Shannon's indices (I) ranged from 0.1449 ± 0.2167 to 0.2204 ± 0.1769 in Holstein cattle and from 0.1481 ± 0.2359 to 0.2328 ± 0.2678 in the case of local cattle, from 0.1034 ± 0.2058 to 0.1604 ± 0.2110 in goats and from 0.2938 ± 0.3016 to 0.3058 ± 0.3004 for sheep. The highest and the lowest of Shannon's indices (I), among the 16 populations, were related to goats (2) and sheep (2) respectively. The mean observed number of alleles (Na) ranged from 1.2333 ± 0.4265 in goats (3) to a maximum of 1.8167 ± 0.3902 in the Holstein Breed (2). The mean Nei's gene diversity (H) ranged from 0.0655 in Goat (2) to 0.2076 in Sheep (2) (Table 2). Values of the effective number of alleles (Ne) were less than those for (Na) with regard to every population and ranged from 1.1085 ± 0.2624 in Goat (2) to 1.3673±0.4050 in Sheep (2). The mean Nei's gene diversity (H) ranged from 0.0655 in Goats (2) to 0.2076 in Sheep (2) (Table 3). Among the 16 populations, the mean coefficient of gene differentiation (Gst) was 0.3615, indicating 19.42% of the total genetic diversity within the populations. Based on the Gst value, the mean estimated number of gene flow (Nm) between populations was found to be 0.8833 (Table 3). The UPGMA dendrogram that was generated represented the genetic relationship among 275 individuals (Fig. 4). In total, 35 (100 to 3100 bp) and 25 (170 to 3000 bp) fragments in PCR products were indicated when, the two primers, $(AG)_9C$ (Fig. 2) or $(GA)_9C$ (Fig. 3) were used respectively. Generally, most of the fragments were common in all populations, but differed in their frequency. Furthermore, in the patterns, few fragments were rarely found that seemed specific, and were only observed in one species with primer $(AG)_9C$. However, because of low frequency of these fragments, it was not possible to use them as markers for distinguishing between species (Table 4). ISSR marker profile produced by primer $(GA)_9C$ on agarose gel

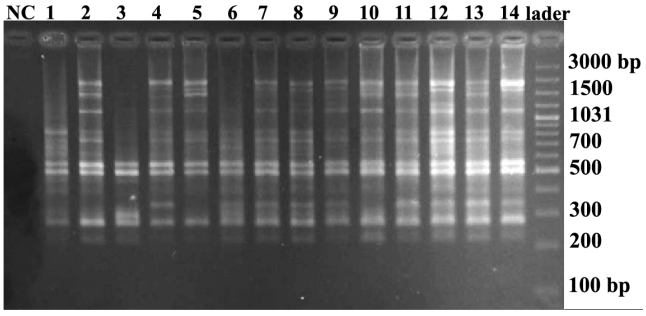

Figure 1. ISSR marker profiles of 14 individuals of sheep (2) population generated by primer $(GA)_9C$ in 2% agarose gel. NC is negative control, 1-14 are animals and ladder is size marker (3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 base pair).

Table 3. Overall genetic variability across all the populations.

Sample size	Observed No. of alleles	Effective No. of alleles	Nei's gene diversity	Shannon's information index	Ht	Hs	Gst	Nm*	No. of polymorphic loci	polymorphic loci (%)
16	2.0000 (0.0000)	1.2836 (0.2481)	0.1942 (0.1429)	0.3205 (0.2000)	0.1909 (0.0195)	0.1219 (0.0075)	0.3615	0.8833	60	100

Nm = estimate of gene flow from Gst. E.g., Nm = 0.5 (1 -Gst)/Gst.

Table 4. Percentage of specific bands were observed in one species with primer $(AG)_9C$.

	260-300(bp)	2110-2200(bp)	2210-2300(bp)	2810-3100(bp)	2710-2800(bp)	2610-2700(bp)	2510-2600(bp)
Goat	0	0	0	0	0	0	0
Holstein	3.41	0	0	0	3.41	2.27	1.14
Local cattle	0	1.75	5.26	8.77	0	0	0
Sheep	0	0	0	0	0	0	0

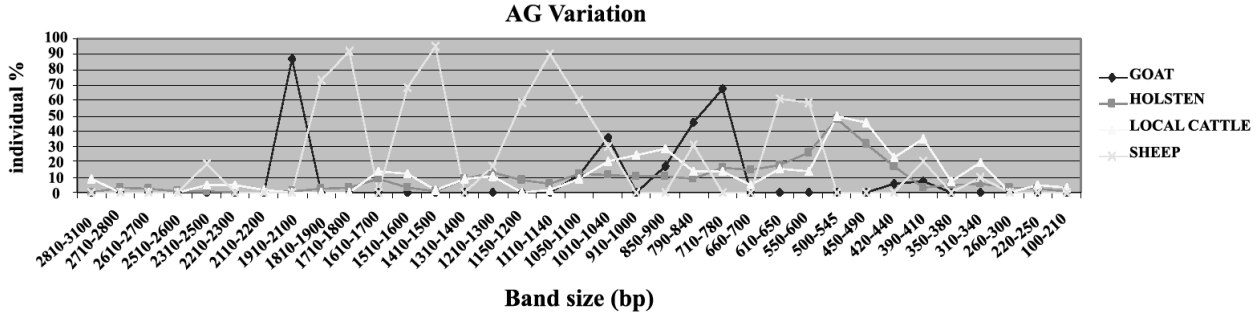

Figure 2. 35 (from 100 to 3100 bp) fragments in PCR products were indicated using $(AG)_9C$.

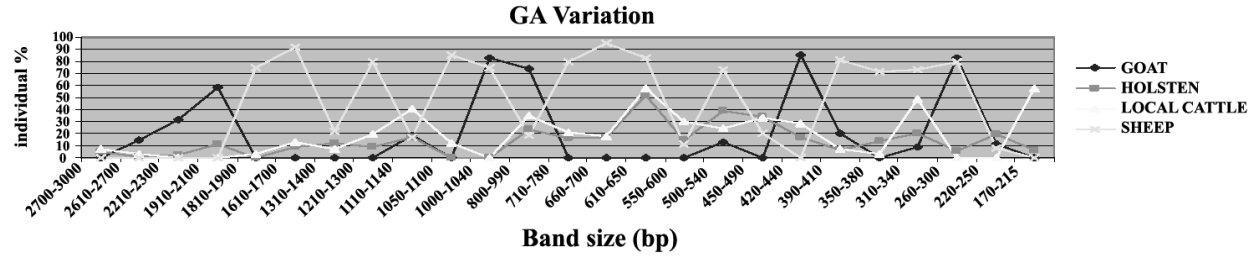

Figure 3. 25 (from 170 to 3000 bp) fragments in PCR products were indicated using $(GA)_9C$.

Table 5. Polymorphism information content (PIC) values were obtained for ISSR primers.

Population	Holstein (1)	Holstein (2)	Holstein (3)	Holstein (4)	Local cattle (1)	Local cattle (2)	Local cattle (3)	Local cattle (4)
$(AG)_9C$	0.1211	0.1155	0.0864	0.0944	0.1222	0.1145	0.1952	0.1517
$(GA)_9C$	0.1301	0.1468	0.1729	0.1759	0.1660	0.1921	0.1442	0.1751
Population	Local cattle (5)	Goat (1)	Goat (2)	Goat (3)	Goat (4)	Sheep (1)	Sheep (2)	Sheep (3)
$(AG)_9C$	0.1454	0.0987	0.1639	0.1952	0.1517	0.2623	0.2687	0.2477
$(GA)_9C$	0.2297	0.2182	0.2013	0.1442	0.1751	0.3468	0.3254	0.3489

is shown in Figure 1. Polymorphism information content (PIC) values were obtained for ISSR analysis. PIC values ranged from 0.1301 at Holstein (1) to 0.3489 at Sheep (3) for $(GA)_9C$ and from 0.0864 at Holstein (3) to 0.2687 at Sheep (2) for $(AG)_9C$ (Table 5). The Nei's original measures of genetic identity and genetic distance ranged from 0.004 to 0.9956 (Table 6). The haplotype analysis was calculated in each species (Tables 8, 9 and 10). As Tables illustrate, the haplotype frequencies based on these two markers were different in

Table 6. Nei's Original Measures of Genetic Identity and Genetic distance.

Population	1	2	3	4	5	6	7	8
1	0	0.9821	0.9846	0.976	0.9735	0.969	0.9347	0.9472
2	0.0181	0	0.9897	0.9891	0.9686	0.9691	0.9517	0.9675
3	0.0155	0.0104	0	0.9895	0.9752	0.9805	0.9436	0.9655
4	0.0243	0.0109	0.0106	0	0.9636	0.9783	0.9527	0.9692
5	0.0269	0.0319	0.0252	0.0371	0	0.9574	0.9526	0.9362
6	0.0314	0.0211	0.0197	0.022	0.0435	0	0.9489	0.9683
7	0.0675	0.0495	0.058	0.0485	0.0486	0.0525	0	0.9423
8	0.0543	0.033	0.0351	0.0313	0.0659	0.0322	0.0595	0
9	0.0177	0.0305	0.0328	0.0355	0.0242	0.0411	0.067	0.0578
10	0.0949	0.072	0.0701	0.07	0.1073	0.0855	0.1276	0.0807
11	0.0877	0.0663	0.065	0.0656	0.0934	0.0773	0.116	0.0711
12	0.0925	0.0718	0.0686	0.0704	0.1054	0.0818	0.1259	0.0768
13	0.0854	0.0584	0.0573	0.0533	0.0941	0.0707	0.1084	0.0662
14	0.152	0.1365	0.1334	0.1258	0.174	0.1423	0.1691	0.1314
15	0.1467	0.1281	0.01264	0.1186	0.1662	0.1321	0.1626	0.1214
16	0.1506	0.1355	0.1306	0.1223	0.174	0.1354	0.169	0.1285
Population	9	10	11	12	13	14	15	16
1	0.9824	0.9094	0.916	0.9117	0.9181	0.859	0.8636	0.8602
2	0.97	0.9305	0.9359	0.9307	0.9433	0.8724	0.8797	0.8733
3	0.9677	0.9323	0.9371	0.9337	0.9443	0.8751	0.8812	0.8776
4	0.9651	0.9324	0.9365	0.932	0.9481	0.8818	0.8882	0.8849
5	0.9761	0.8982	0.9109	0.9	0.9102	0.8403	0.8469	0.8403
6	0.9597	0.918	0.9256	0.9215	0.9318	0.8673	0.8763	0.8734
7	0.9352	0.8802	0.8905	0.8817	0.8973	0.8445	0.8499	0.8445
8	0.9438	0.9225	0.9313	0.9261	0.9359	0.8768	0.8856	0.8794
9	0	0.9131	0.925	0.9125	0.9238	0.8639	0.8694	0.8634
10	0.0909	0	0.9931	0.9908	0.9934	0.8517	0.8572	0.852
11	0.078	0.0069	0	0.9889	0.9903	0.8569	0.8629	0.8569
12	0.0916	0.0092	0.0112	0	0.9907	0.8512	0.8577	0.8505
13	0.0792	0.0066	0.0098	0.0094	0	0.8604	0.8661	0.8599
14	0.1463	0.1605	0.1544	0.1611	0.1504	0	0.9943	0.9956
15	0.1399	0.154	0.1474	0.1535	0.1438	0.0058	0	0.9947
16	0.1468	0.1602	0.1545	0.1619	0.151	0.004	0.0053	0

each species. At sheep haplotype 1 was the most frequent haplotype (0.267) and haplotype 9 was the least one (0.033) for $(AG)_9C$ but for $(GA)_9C$, haplotype 2 was the highest (0.217) and haplotype 7 was the least frequent one (0.017). For $(AG)_9C$ at goat, the highest and the lowest frequencies were related to haplotype 1 (0.529) and haplotype 5 (0.028) respectively and for $(GA)_9C$ the highest and the lowest ones were haplotype 1 (0.443) and haplotype 6 (0.014). At cattle haplotype 3 was the most frequent haplotype (0.242) and haplotype 2 was the least one (0.007) for $(AG)_9C$ whereas for $(GA)_9C$ haplotype 4 was the highest (0.255) and haplotype 9 was the least frequent one (0.021).

DISCUSSION

Genetic variation is a basic requirement for animal breeding, whereas a high genetic variation is needed for genetic improvement of domestic animals. In recent years, genetic markers are increasingly used for the study of genetic diversity. Moreover, the polymorphism determined by these markers is one of the valuable parameters for study of populations and understanding of their genetic differences. The high reproducibility of ISSR markers may be because of using longer primers and higher annealing temperature than those used for RAPD. Based on its unique characters, ISSR technique can detect more genetic loci than

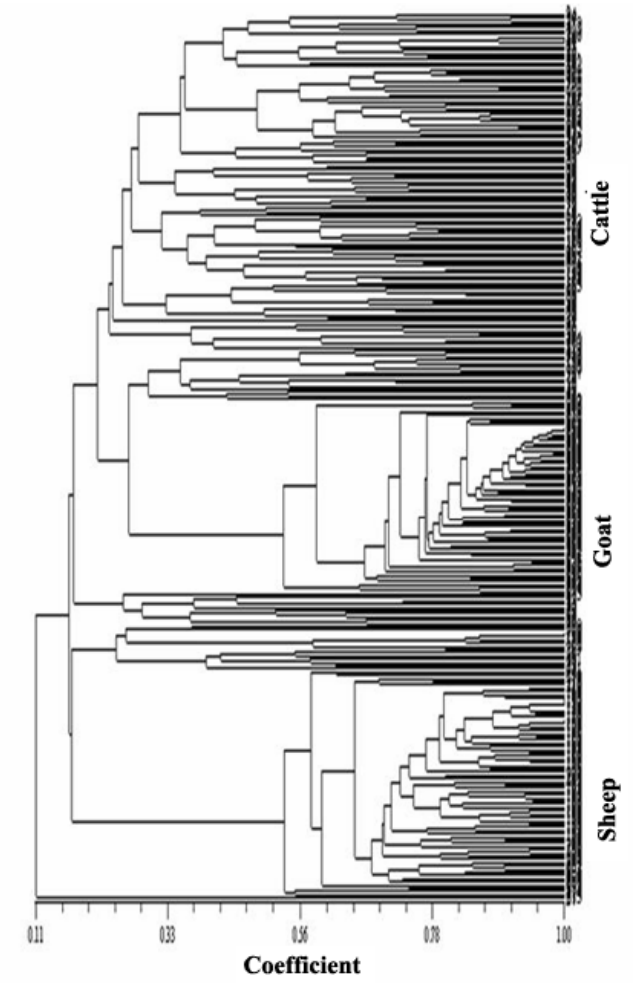

Figure 4. Dendrogram illustrating genetic relationships among 275 individuals in genetic diversity study generated by UPGMA cluster analysis from 60 ISSR bands produced by 2 primers.

isozyme and has higher stability than RAPD (Zietkiewicz *et al*., 1994). Our work is the first application of this method to compare the native populations of cattle, goats and sheep. The experimental results of this study will provide evidence for the reliability and usefulness of ISSR markers, to estimate genetic diversity within and between native livestock populations. The average amount of heterozygosity that was calculated in this study was almost high (0.5356) (Table 7) and the mean value of Ht (Nei's genetic diversity among populations) and Hs (Nei's genetic diversity within subpopulations) and the mean coefficient of gene differentiation (Gst) in this study were 0.19, 0.12 and 0.36 respectively. In comparison, study of the genetic diversity in color types of Markhoz goat populations using ISSR markers determined that total heterozygosity (Ht) and heterozygosity within color types (HS) were 0.33, 0.30 respectively. The coefficient of population differentiation (Gst) was 0.91, indicating that the genetic variation between color types is small part of the total heterozygosity (Moradi *et al*., 2007). Our primers were different from the primers used in the study of Markhoz goat populations and so the authors believe that it is possible the sequence of ISSR markers used in each study have effect on the value of Gst. Our result showed that based on the Shannon's information index, genetic diversity in sheep populations is higher than the others and that the goat populations had the lower one; however, there is no significant diversity within species (Table 2). In all of sixteen populations genetic diversity wasn't high and it can be related to the low number of markers that we used in this study. Further studies should be performed in future with a greater number of

Table 7. Observed, expected and average of homozygosty and heterozygosity.

Primers	Obs_Hom	Obs_Het	Exp_Hom	Exp_Het	Ave_Het
P01	0.0000	1.0000	0.4505(0.0490)	0.5495(0.0490)	0.5321(0.0310)
P02	0.0044(0.0222)	0.9956(0.0222)	0.4358(0.0475)	0.5642(0.0475)	0.5406(0.0227)
P01, P02	0.0148(0.1012)	0.9852(0.1012)	0.4362(0.0617)	0.5638(0.0617)	0.5356(0.0280)

Table 8. Frequency of haplotypes based on two markers for sheep.

	Primers																			
	P01									P02									
Haplotype	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	10
Frequency	0.267	0.183	0.100	0.067	0.050	0.167	0.050	0.083	0.033	0.267	0.183	0.100	0.067	0.050	0.167	0.050	0.083	0.033	0.050

Table 9. Frequency of haplotypes based on two markers for goat.

	Primers											
	P01					P02						
Haplotype	1	2	3	4	5	1	2	3	4	5	6	7
Frequency	0.529	0.271	0.129	0.043	0.028	0.443	0.057	0.186	0.214	0.057	0.014	0.029

Table 10. Frequency of haplotypes based on two markers for cattle.

	Primers									
	P01									
	1	2	3	4	5	6	7	8	9	10
Haplotype	0.090	0.007	0.248	0.221	0.110	0.076	0.055	0.041	0.014	0.103
Frequency	P02									
	1	2	3	4	5	6	7	8	9	
	0.034	0.145	0.207	0.255	0.131	0.097	0.048	0.028	0.021	

ISSR markers in order to obtain more accurate results. Genetic variation in Bovinae, quantity and quality of amplified DNA fragments, using ISSR-PCR method, in Mongolian yaks (*Bos grunniens*) and fifteen cattle breeds were evaluated. Results showed that 53 fragments out of 55 were polymorphic and there were some differences in quantity and quality of observed fragments in yaks and cattle breeds. Generally, more than 90% of the fragments were common in all investigated breeds, but differed in their frequency (Ahani Azari *et al*., 2007). However our results showed 60 polymorphic fragments with some differences in quantity and quality of observed fragments in those three species. We found that in most fragments, sheep had the highest frequencies rather than the others. The haplotype analysis of ISSR markers revealed that, some of them to be significantly less frequent in each species. Furthermore, in these three species some unique haplotypes were introduced in this paper. For $(AG)_9C$ haplotype 10 was a specific haplotype at cattle, on the other hand, haplotype 10 is a particular haplotype at sheep for $(GA)_9C$. The ISSR marker data were collected and used to analyze genetic diversity through cluster analysis. A UPGMA tree was prepared using the NTSYS-PC sub-program "Simqual" which used "Sham" coefficient to establish genetic relationships at the molecular level. The selected genotypes were differentiated and placed as individual entities for ISSR marker system-generated cluster trees. In the ISSR-based cluster analysis, some similar groupings were obtained as represented in Figure 1. Each species carrying indigenous pedigrees like (goat: 1, 2, 3 and 4) (sheep: 1, 2, and 3) (local cattle: 1, 2, 3, 4, 5 and Holstein: 1, 2, 3, 4) were placed in one group and as we expected these three species were placed separately in different groups based on their genetic structures and also there were no more differences between local and Holstein cattle. Comparison of PIC values for these two ISSR markers (a parameter associated with the discriminating power of markers) that showed the PIC values for $(GA)_9C$ was higher than $(AG)_9C$ in all of those populations (Table 5) and so, indicated better resolving power of the $(GA)_9C$ in accessing genetic variation in these livestock. It was concluded that the low level of genetic diversity in cattle breeds may be the result of common use of Holstein sperms in A.I. for increasing milk production and creating new high performance breeds. On the other hand, in sheep populations, the low level of genetic diversity was related to inbreeding in different herds (Table 6).

Generally speaking, genetic relationship among native livestock populations is a priority for managing farm animal genetic diversity. This study is the first of characterization of genetic structure of cattle, goat and sheep populations. As the results illustrate both of these two markers were polymorphs. A greater under-

standing of the potential of native species is necessary for supporting long-term genetic improvement and so it is suggested that this marker system was found to be efficient in discriminating each genotype at the molecular level and can be used for genetic diversity analysis for livestock animals.

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