

STUDY OF GENETIC DIVERSITY OF IRANIAN INDIGENOUS BUFFALO POPULATIONS USING MICROSATELLITE MARKERS

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Indigenous buffalo breeds represent a unique genetic resource, and understanding their variability, population structure and breeding units is important for their sustainable conservation. In the present study the genetic structure of Iranian buffalo populations was analyzed using ten microsatellite markers. Two hundred hair samples were collected and DNA was extracted using modified salting out method. After Polymerase Chain Reaction (PCR), the PCR products were electrophoresed using 9% polyacrylamide gel. Fifty- nine alleles were observed for all the loci. The average number of alleles was 5.90 and the effective average number of alleles was 4.86. The high level of mean heterozygosity index between three populations indicate that the genetic diversity is high in within and between populations. The mean of polymorphism information content (PIC) value for all loci was 0.70. The F_{ST} value for the total loci was 0.01, indicating a very low level of genetic structure among populations. The genetic structure AMOVA analysis showed that about 3% of the total genetic variation was explained by population differences and 97 percent was corresponded to differences among individuals. The obtained results at the present study indicated that characterization of genetic diversity by employing molecular tools is a prerequisite in developing strategies for conservation and utilization of buffalo genetic resources.

Keywords: AMOVA analysis, genetic diversity, Iranian buffalo, microsatellite markers, PCR

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INTRODUCTION

The need to maintain and improve local genetic resources has been recognized as a priority. The maintenance of genetic diversity in livestock species requires the adequate implementation of conservation priorities and sustainable management programs, which should be based on comprehensive information regarding the structure of the populations, including sources of genetic variability among and within breeds and populations (KEVORKIAN *et al.*, 2010).

In Iran, there are about 234 thousand buffalo (FAOSTAT, 2009) with a vast genetic variation comprising of three ecotypes that all of them are classified as riverine type. According to climate conditions Iranian buffaloes can be classified in three main populations: i) Azarie population (Western & Eastern Azarbaijan); ii) North population (Guilan and Mazendaran); iii) Khuzestani population (Khoozestan) (NASERIAN and SAREMI, 2010). Buffaloes have an effective and important role in the economy of rural families in the areas that they are bred due to their abilities for producing milk, meat, and draft power (NASERIAN and SAREMI, 2010).

Molecular methods such as microsatellites, are useful tools to study the genetic variations. Microsatellites are stable, polymorphic, easy to analyze and occur regularly throughout an animal genome. The availability of microsatellites markers has facilitated genetic linkage studies; including mapping and searching for genes affecting productive traits as well as estimating genetic diversity in farm animals (GOLDSTEIN and SCHLÖTTERER, 1999, HOSHINO *et al.*, 2012). Several studies of genetic relationships between buffalo breeds using the microsatellite method were reported previously (ÁNGEL-MARÍN *et al.*, 2010; BARKER *et al.*, 1997; MIRHOSEINIE *et al.*, 2005; MOIOLI *et al.*, 2001; NAVANI *et al.*, 2002; ZHANG *et al.*, 2011).

The purpose of the present study was to evaluate the genetic diversity of three buffalo populations in Iran based on microsatellite markers and to estimate the genetic relationships among these three populations for better utilization in breeding programs.

MATERIALS AND METHODS

Sample collection

Two Hundred animals classified into three groups were sampled as: i. Azarie buffalo represented by 70 animals located in Azarbayegan and Ardebil provinces. ii. Khuzestani buffalo represented by 70 in Khuzestan province. iii. North buffalo, represented by 60 animals belonging to various farms in Guilan and Mazandaran provinces. Hair pulps samples were taken from tail and they were stored at 20°C. DNA was extracted by optimized and modified salting out technique (MILLER *et al.*, 1988). The quantity and quality of the extracted DNA was determined by a spectrophotometric method based on absorbance at 260 and 280 nanometer respectively (GALLAGHER and DESJARDINS, 2007).

Genotyping and microsatellite loci

The microsatellite markers used in this study were chosen according to published studies on microsatellite genetic variation in water buffalo populations (BARKER *et al.*, 1997; KATHIRAVAN *et al.*, 2009; MOIOLI *et al.*, 2001; NAVANI *et al.*, 2002; ZHANG *et al.*, 2007). A total of ten heterologous microsatellite loci were chosen for the study to be polymorphic in buffalo. Information on the ten microsatellite investigated is presented in Table 1. The Polymerase Chain Reactions (PCR) was performed in a 15µL mixture containing 0.30 pmol primers, 1X Ampliqon (Taq DNA Polymerase Master Mix Red 2X) consisting of 3 mM MgCl₂, 0.2 U Taq-DNA

polymerase and 100 ng genomic DNA as template. Optimum PCR amplification conditions were determined for each marker separately. PCR products were separated by 9% PAGE (acrylamide:bisacrylamide = 19:1) gel electrophoresis and visualisation was silver staining procedure (SANGUINETTI and SIMPSON, 1994). The Gel-Pro Analyser software (Media Cybernetics), version 4.0, was used to determine the amplified fragment length and assign genotypes to each sample.

Table 1. Characteristics of microsatellite markers tested in Iranian Buffalo populations

Locus	Annealing Temp. (°C)	Size of observed alleles (bp)	Location
BoLA-DRB3	59.5	285, 322, 343, 380	2
BM1818	58	246, 254, 264, 275, 284, 291	23
BM1824	57	280, 184, 189, 194, 198	1
CSSM033	58	147, 151, 159, 166, 176	17
CSSM047	58.5	132, 139, 142, 146, 150, 158, 161	8
ILSTS017	59	120, 124, 127, 132, 137	X
ILSTS033	57	144, 147, 151, 164, 168	12
ILSTS058	58	115, 122, 125, 132, 140, 144, 150	17
ILSTS061	57	142, 144, 148, 157, 161, 165, 170, 178, 180	15
ILSTS089	58	117, 121, 123, 125, 130, 133	5

Statistical analyses

The microsatellite loci were checked for possible allelic stuttering, allele drop out and null alleles in MICRO-CHECKER version 2.2 (VAN OOSTERHOUT *et al.*, 2004). Allele frequency, number of alleles, observed and expected heterozygosity and Wright's F-statistic were calculated using the statistics programs GENALEX 6.0 (PEAKALL and SMOUSE, 2006) and POPGENE3.1 (YEH *et al.*, 1999). Polymorphic Information Content (PIC) was calculated, using Het1.8 software (OTT, 2001). Tests for deviation from Hardy-Weinberg equilibrium (HWE) at each locus for each breed were performed using POPGENE3.1 software (YEH *et al.*, 1999). Standard genetic distance estimates, D_A of TAKEZAKI and NEI (1996) were used to construct dendrogram of phylogenetic relationship tree with the neighbor-joining (NJ) using POPTREE2 package (TAKEZAKI *et al.*, 2010). An analysis of molecular variance (AMOVA) was computed to test significant differences in genetic diversity between Iranian buffalo populations using GENALEX 6.0 (PEAKALL and SMOUSE, 2006).

RESULTS

A total of 200 animals representing three populations of Iranian buffaloes were analyzed using ten microsatellite markers. Microsatellite data were analyzed using software Micro-Checker and none of genotyping errors (allelic stuttering, allele drop out and null alleles) in ten microsatellite loci was observed. After this analysis, microsatellite data were used to estimate the population parameters.

The genetic variability estimates in terms of the observed number of allele (N_a), effective number of allele (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), Hardy–Weinberg equilibrium (HWE), polymorphic information content (PIC), F-statistics (F_{IS} , F_{IT} , and F_{ST}) and Gene flow (Nm) are presented in Table 2. The percentage of polymorphic loci was 100%. N_a varied from four (BoLA-DRB3) to nine (ILSTS061) and a total of 59 alleles were observed with a mean of 5.90 alleles. N_e was distinctively less than the observed value across all the loci and ranged between 1.52 and 8.01 for BoLA-DRB3 and ILSTS061, respectively, with a mean of 4.86.

Table 2. Measures of genetic variation across ten microsatellite loci in Iranian buffaloes.

Locus	N_a	N_e	H_o	H_e	HWE	PIC	F_{IS}	F_{IT}	F_{ST}	Nm
BoLA-DRB3	4.00	1.52	0.39	0.34	0.976 ^{ns}	0.33	-0.17	-0.15	0.01	17.47
BM1818	6.00	5.80	0.98	0.82	0.000 ^{***}	0.80	-0.21	-0.19	0.02	11.54
BM1824	5.00	4.67	0.99	0.79	0.000 ^{***}	0.75	-0.29	-0.26	0.02	10.74
CSSM033	5.00	2.56	1.00	0.61	0.000 ^{***}	0.53	-0.65	-0.64	0.01	46.16
CSSM047	7.00	5.74	0.79	0.83	0.000 ^{***}	0.80	0.03	0.04	0.01	18.99
ILSTS017	5.00	4.50	0.96	0.78	0.000 ^{***}	0.74	-0.24	-0.24	0.00	86.42
ILSTS033	5.00	4.47	0.87	0.78	0.000 ^{***}	0.74	-0.13	-0.12	0.01	22.44
ILSTS058	7.00	5.72	0.98	0.83	0.000 ^{***}	0.80	-0.21	-0.20	0.01	18.78
ILSTS061	9.00	8.01	0.82	0.88	0.000 ^{***}	0.86	0.04	0.05	0.01	22.13
ILSTS089	6.00	5.67	0.94	0.82	0.000 ^{***}	0.80	-0.15	-0.14	0.01	31.32
Mean	5.90	4.86	0.87	0.75		0.70	-0.20	-0.18	0.01	28.60

N_a : Observed number of allele, N_e : Effective number of allele, H_o : observed heterozygosity, H_e : expected heterozygosity, HWE: Hardy–Weinberg equilibrium (ns=not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$), PIC: Polymorphic information content, F_{IS} , F_{IT} , and F_{ST} = F-statistics and Nm: Gene flow.

The genetic analysis showed that Iranian buffaloes have a reasonably high level of diversity as reflected by average heterozygosity estimates. The H_o and H_e varied between 0.39 (BoLA-DRB3) to 1.00 (CSSM033) and 0.34 (BoLA-DRB3) to 0.88 (ILSTS061) respectively across different loci. The overall mean H_o and H_e were found to be 0.87 and 0.75 respectively. PIC was estimated using allele frequencies in each polymorphic microsatellite locus (Table 2). The overall mean PIC was estimated to be 0.70 and it varied from 0.33 (BoLA-DRB3) to 0.86 (ILSTS061).

The mean values of F_{IS} (within population inbreeding) and F_{IT} (within-population inbreeding estimate), 0.20 and -0.18 respectively indicating a very low degree of genetic structure among populations. F_{ST} indicates the degree of genetic differentiation between populations. The average value for the total F_{ST} loci in the analyzed population was 0.01, indicating a very low degree of genetic structure among three populations. The mean estimate of gene flow (Nm) was found to be 28.60 and the values for individual loci varied considerably and ranged from 10.74 (BM1824) to 86.43 (ILSTS017). As can be seen from Table 2, observed heterozygosity has obtained lower than expected heterozygosity for CSSM047 and ILSTS061

loci, in addition, the values of inbreeding FIS and FIT were positive for these two loci that may be due to sampling variance or genetic drift.

To check for HWE at 10 loci across three populations, a total of 30 tests were conducted. Significant departures from HWE were observed for all loci except BoLA-DRB3 locus ($P < 0.001$), reflecting an excess of heterozygote individuals than homozygote individuals in the population and also negative inbreeding values confirm this case. The AMOVA analysis showed that 3 percent of the genetic variation among Iranian buffaloes is attributed to among populations compared with 97 percent due to variation within populations. A higher variation within than among Iranian buffalo populations suggests high levels of gene flow.

Standard genetic distance (TAKEZAKI and NEI, 1996) of the three populations is listed in Table 3. The greatest and lowest genetic distance were between Azarie and North populations (0.0047) and Azarie and Khuzestani populations (0.037), respectively. The dendrogram illustrating the relationship between these populations is shown in Figure. 1. Azarie and Khuzestani populations clustered the most closely and North population located further distance than two other populations.

Table 3. Matrix of NEI's (1978) standard genetic distance among three buffalo populations.

Population	Azarie	Khuzestani	North
Azarie	0.000		
Khuzestani	0.037	0.000	
North	0.047	0.043	0.000

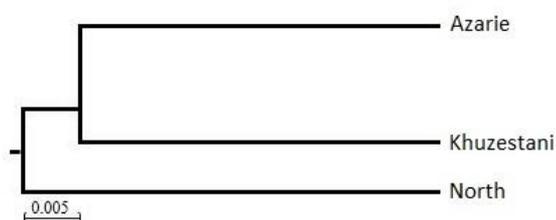


Figure 1. Neighbor-joining (NJ) tree of Iranian buffalo populations

DISCUSSION

This paper provides the description of genetic variability using molecular markers in the Iranian buffalo populations. The genetic analysis of ten microsatellite loci showed that Iranian buffaloes have a reasonably high level of diversity. The different measures of genetic variation

showed that all of the studied loci were highly informative, indicating high polymorphism across the loci, thus suggesting suitability of these markers for the study of genetic diversity in buffalos. Suitability of these markers was further supported by the fact that the number of alleles for each marker was higher than the minimum number of alleles (four) recommended for microsatellite markers to be used in the estimation of genetic distance in order to reduce the standard error (MISHRA *et al.*, 2012).

These populations of buffalo showed low effective number of alleles than the observed number. This is due to very low frequency of most of the alleles at each locus and very few alleles might have contributed to the major part of the allelic frequency at each locus (AMINAFSHAR *et al.*, 2008).

The obtained results from heterozygosity indicated that there are desirable genetic variation in buffalo populations. The mean expected heterozygosity was higher than those reported for Asian river buffalo (0.58) (BARKER *et al.*, 1997), Chinese buffalo (0.40) (YANG *et al.*, 2011), Greek (0.18) and Italian buffalo (0.17) (MOIOLI *et al.*, 2001), Iranian buffalo (0.71) (MIRHOSEINIE *et al.*, 2005) but lower than those of Anatolian buffalo (0.81) (SOYSAL *et al.*, 2010) and African buffalo (0.76) (VAN HOOFT *et al.*, 2000).

The polymorphism information content is a parameter indicative of the degree of informativeness of a marker. Genetic markers with PIC values more than 0.5 are reckoned as distinctly informative in population genetic studies (BOTSTEIN *et al.*, 1980). Consequently, With the exception of BoLA-DRB3, all the nine microsatellite loci used in the present study can be considered useful for the evaluation of genetic diversity in buffalo populations. The mean PIC obtained in this study is more than that reported in other buffalo breeds (AMINAFSHAR *et al.*, 2008; ÁNGEL-MARÍN *et al.*, 2010; KATHIRAVAN *et al.*, 2009).

Significant deviations from HWE were observed at many investigated loci in Iranian buffalo populations. Departure from HWE is mostly due to heterozygote excess which may result from one or more of the following reasons: association of loci with some gene of economics importance, migration and high mutation rate of microsatellite (AMINAFSHAR *et al.*, 2008).

Mean values of inbreeding within the populations were negative, indicating the absence of inbreeding within the populations. This result is confirmed by the high values of heterozygosity observed for all the markers in all the populations. The F_{ST} value was low for all loci (0.01), indicating a heterozygote excess which implies a low genetic differentiation between populations. This is expected because of the animals' exchange policy between the different regions over Iran. Compared with other studies on genetic diversity using microsatellites in *Bubalus bubalis*, low F_{ST} values were found, indicating little inbreeding and low genetic structure (ÁNGEL-MARÍN *et al.*, 2010; MOIOLI *et al.*, 2001; ZHANG *et al.*, 2011). The high values of Nm (28.60) point towards immigration and expected gene flow of buffaloes from other parts of the country especially the use of Khuzestani buffaloes which have higher milk yield.

Although abundant genetic variation was observed, the population differentiation in Iranian buffalo was very weak. Only 3% of the genetic variation was explained by population differences. These values were far lower than those from other genetic diversity studies, e.g. 16.8% for Southeast Asian swamp buffalo (BARKER *et al.*, 1997), 5.7% for three river buffalo populations from Italy, Greece and Egypt (MOIOLI *et al.*, 2001), 2.8% for Chinese domestic buffalo (ZHANG *et al.*, 2007) and 6.2% for Turkish water buffalo populations (GARGANI *et al.*, 2010). The low degree of genetic differentiation in Iranian buffalo might result from the

following two reasons: (i) in Iran, buffalo are reared mostly by the small-holder farmers for draught purpose without strict and systematic selection on the other economical traits; (ii) use some of these populations among other populations might enable frequent genetic exchanges.

The cluster by populations confirms the low genetic distance between populations. This indicates genetic mix and a minimal differentiation between populations, possibly because some of these populations were founders of animals to different areas of the country.

CONCLUSIONS

We have provided the comprehensive insight into the genetics of the Iranian riverine buffaloes using microsatellite markers. The results indicate abundant genetic variation and the absence of inbreeding within the populations. Due to the crisis of a sharp decline in the number of the Iranian buffaloes, our data will be useful for the development of rational breeding in the dairy buffalo industry and conservation strategies for Iranian buffalo.

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**ISTRAŽIVANJE GENETIČKE RAZNOVRSNOSTI POPULACIJA IRANSKIH
DOMAĆIH BIVOLA KORIŠĆENJEM MIKROSATELITSKIH MARKERA**

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Izvod

Autohtone rase bivola predstavljaju jedinstven genetički resurs, a poznavanje njihove varijabilnosti, strukture populacije i oplemenjivačkih mogućnosti važno za njihovu održivu konzervaciju. U ovoj studiji analizirana je genetička struktura iranskih populacija bivola, korišćenjem deset mikrosatelitskih markera. Prikupljeno je dve stotine uzoraka dlake i DNK je ekstrahovana korišćenjem modifikovane metode soljenja. Urađena je elektroforeza PCR produkata korišćenjem 9% poliakrilamidnog gela. Ukupno je utvrđeno 59 alela na svim lokusima. Prosečan broj alela bio je 5.9, a efektivan prosečan broj iznosio je 4.86. Visok nivo srednjeg indeksa heterozigotnosti između tri populacije ukazuje na to da je genetička raznolikost visoka unutar i između populacija. Srednja vrednost PIC za sve lokuse bio je 0.70. Vrednost FST za sve lokuse bila je 0.01, što ukazuje na veoma nizak nivo genetičke strukture između populacija. Analiza genetičke strukture AMOVA, pokazala je da je oko 3% ukupne genetičke varijacije objašnjeno razlikama u populaciji, a 97% razlikama između individua. Dobijeni rezultati ukazuju da je karakterizacija genetičke raznolikosti primenom molekularnih metoda preduslov u razvoju strategija za očuvanje i korišćenje genetičkih resursa bivola.

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