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Isolation and Characterization of Microsatellite Markers from Endangered Species (*Camelus bactrianus*)

Research Article

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ABSTRACT

Iranian bactrian camel (*Camelus bactrianus*) is an endangered livestock breed with distribution in northwest of Iran. Microsatellites are a powerful marker for animal genetic and cell line identification and population genetic study. In this study, after producing more than 40 *Camelus bactrianus* fibroblast cell lines, microsatellites loci from the genome of Iranian *Camelus bactrianus* cell lines were identified using polymerase chain reaction (PCR)-based isolation of microsatellite arrays (PIMA) methods for genetic resources studies. PIMA is a relatively simple method which avoids not only library construction, but also radioactivity manipulation. Four polymorphic microsatellite loci (IBRC01 to IBRC04) were identified. Polymorphism of these new loci was assessed in 40 samples of Iranian bactrian camel. Number of alleles ranged from 3 to 5, observed and expected heterozygosity varied from 0.625 to 0.71 and from 0.642 to 0.807 and Shannon index from 0.89 to 1.603, respectively. The phylogeny analysis of all sequences indicated that breeds and species were grouped according to their geographic locations except a few sequences. These newly isolated polymorphic microsatellite markers would be useful tools for conservation genetic resources and population genetic studies and assessing genetic variations to establish a conservation strategy of this endangered species.

KEY WORDS *Camelus bactrianus*, conservation, microsatellite, PIMA.

INTRODUCTION

Bactrian camel (*Camelus bactrianus*) is better known as being the type of camel with two humps. The bactrian camel was domesticated in 2500 BC, probably in Northern of Iran, Northeast Afghanistan, or Southwestern Turkmenistan (Bulliet, 1975). Iranian Bactrian camel lives in different regions of Iran (Ardebil province). The bactrian camel is one of the most adaptable animals in the world that being able to withstand temperatures from 40 ˚C in the summer to -40 ˚C in the winter (Shahkarami *et al.* 2012). The most noticeable features of the bactrian camels are their two humps with an average height of 213 cm at the top of the hump. The bactrian camel is strong in constitution, with well-developed musculature. Their even-toed feet help to cross the rocky deserts of Asia and travel well through snow or sand. Live body weight can range from 300 to 690 kg, with males often being much larger and heavier (average 446 kg) than females (average 394 kg) (Potts, 2005). Iranian *Camelus bactrianus* has experienced a dramatic

decrease in the last decade. Nowadays, their numbers are less than 100 heads in Iran and they are an endangered breed in the first homeland, unfortunately. Genetic variation and characterization are the initial step in developing conservation and breeding programs. The repeat sequence content of the camelid genomes (30.4% in bactrian camel, 32.1% in alpaca and 28.4% in dromedary) was 10% lower than cattle and humans. This is likely one of the reasons that the Bactrian and dromedary camel have a smaller genome size than other mammals, for example, human (2.9 Gb), mouse (2.5 Gb), horse (2.7 Gb) and cattle (2.9 Gb) (Fitak *et al.* 2016; Wu *et al.* 2014). Microsatellites or short tandem repeat (STR) have been widely used as DNA markers in the studies of population structure, identification of individuals, paternity testing, inference of demographic processes and conservation of genetic resources (Ellegren, 2004; Zane *et al.* 2000). The main advantages of the microsatellite markers are that, they are polymorphic and reproducible (Abdul-Muneer, 2014). There are several different methods for isolating microsatellite markers. PCR-based isolation of microsatellite arrays (PIMA) is a relatively simple and moderate in time and cost, microsatellite isolation technique which avoids not only library construction but also radioactivity manipulation (Lunt *et al.* 1999; Sadder *et al.* 2015). This approach is based on random amplified polymorphic DNA (RAPD) process, but investigates microsatellite arrays by repeat-specific PCR rather than radioactive hybridization (Ma *et al.* 2009). There were few studies on genetic structure, isolation and characterization of microsatellite markers and population variation in Iranian *Camelus bactrianus*. In most studies of population genetics that has been done in bactrian camel, the markers identified in other species of the family camelidae have been used. These markers may not show specific allele and genetic diversity exactly (Chuluunbat *et al.* 2014). Due to the limited research on the identification of specific markers in Bactrian camel, it appeared necessary to isolate and develop these kinds of genetic markers in *C. bactrianus*. Here, We used genome of cell lines, which are characterized, and cryopreserved in nitrogen tank as a unique and valuable source of endangered breed (Elyasi Gorji *et al.* 2017). We report the isolation and characterization of the first polymorphic microsatellites markers for Iranian Bactrian camel, which can be used for various population genetic researches, parentage test and cell line identifications.

MATERIALS AND METHODS

Ear margin tissues (about 1 cm^2 in size) were sampled with an "O" shape Ear Notcher (AESCULAP Co.) from the bactrian camel for fibroblast cell line establishment. DNA was purified by traditional phenol-chloroform protocol and ethanol precipitation. The quality and quantity of genomic DNA were determined with a NanoDrop spectrophotometer.

The isolation of microsatellite markers began with a set of 14 RAPD primers (Chiang *et al.* 2008; Lin *et al.* 2008). PCR amplifications were performed in a 50 µL reaction volume containing 1 × PCR buffer, 2 m*M* $MgCl_2$, 200 µ*M* of dNTPs, 20-100 ng of the template DNA, 10 p*M* of each primer and 1 unit of Taq DNA polymerase (invitrogen). Amplifications were conducted on a Bio-rad My Cycler Thermal Cycler using the following conditions: initial denaturing at 94 ˚C for 5 min, 45 cycles of 94 ˚C denaturing for 50 s, 35-39 ˚C annealing for 1 min, 72 ˚C extension for 1 min, and 72 ˚C final extension for 10 min. RAPD-PCR products were size-selected to preferentially obtain small fragments (500-1000 bp).

Selected fragments were purified from 1.5% agarose gel using clean up kit (Fermentas) and ligated into a PTZ57R TA cloning vector (Fermentas) according to manufacturer's instructions, the ligation mixture was transformed into competent *Escherichia coli* cells to obtain the genome libraries. Positive clones were screened and plasmid DNA was isolated using the high-speed Plasmid Mini Kit (IBRC). The colonies were identified by colony PCR with M13 primers and verified by gel electrophoresis on a 2% agarose gel. Purified PCR products were directly sequenced at MACRO GEN Co., Ltd. Primer pairs were designed for candidate loci using Oligo, Primer 3 and Primer-Blast (Rozen and Skaletsky, 2000); annealing temperature was adjusted to 57-60 ˚C and fragment size to separate categories, to facilitate multiplex PCR and minimize overlapping of fragment sizes during visualization. Amplification conditions were set up for each marker and polymorphism assessment was performed on 40 heads of Iranian Bactrian camel. Reactions were performed in a 15 µL reaction volume containing 10-100 ng of genomic DNA, 1x PCR buffer, 2 m*M* $MgCl_2$, 200 µ*M* dNTP, 0.2 µ*M* of each primer and 1 unit of Taq DNA polymerase (IBRC, MBE0100).

PCR reactions were as follows: initial denaturing at 94 ˚C for 5 min, 30 cycles of 94 ˚C denaturing for 30 s, annealing for 30 s, 72 ˚C extension for 45 s, and 72 ˚C final extension for 10 min (Table 1). The electrophoresis of PCR products were done on 8% denaturing acrylamide gels and visualized by silver nitrate staining. The microsatellite length and the allele frequencies were estimated using GELPRO analyzer software (version 3.1) (Creste *et al.* 2001).

Allele frequencies, number of alleles, expected (H_e) and observed (H_o) heterozygosities, Shannon index, test for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were calculated using the POPGENE (Yeh *et al.* 1999) and GENEPOP (Rousset, 2008) programs.

Table 1 Random amplified polymorphic DNA (RAPD) primers characterization and number of cloned fragments

Primers	Sequence (5' to 3')	Annealing temp (˚C)	Number of bands	Sequence length (bp)
GC-10	GCC GTC CGA G	39	1	609
G-2	TCG CGA GCT G	35	2	510, 711
GT-10	GTG ATC GCA G	37	1	722
OPB-08	GTC CAC ACG G	37	1	430
OPF-05	CCG AAT TCC C	37	2	575, 716
OPA-03	AGT CAG CCA C	37	1	531
OPA-04	AAT CGG GCT G	37	2	783, 1196
OPA-11	CAA TCG CCG T	37	1	615
OPB-02	TGA TCC CTG G	36	1	545
OPB-05	TGC GCC CTT C	36	2	509, 856
OPB-07	GGT GAC GCA G	36	1	462
OPB-10	CTG CTG GGA C	36	1	717
OPB-14	TCC GCT CTG G	35	-	-
IBRD-01	AAC CGC CTG A	36	1	576

All homologous sequences from bactrian, dromedary camel and alpaca were identified from gen bank (NCBI). Iranian Bactrian camel sequences were aligned with these homologous sequences. Phylogenetic tree was constructed from the aligned sequences, by neighbor-joining and unweighted pair group method with arithmetic mean (UPMGA) methods (Tamura *et al.* 2011).

RESULTS AND DISCUSSION

In the first step of PIMA method, thirteen RAPD primers out of fourteen primers had clear and sharp bands after RAPD-PCR reactions. The OPB-14 band was not clear and GT-10, GC-10, OPF-05 and G-2 bands were clear and thick. Twenty four sharp fragments selected after RAPD-PCR reactions were ligated into the PTZ57R TA cloning vector and transformed. Seventeen fragments out of twenty four fragments were cloned as well. Among these fragments, three of them were larger than 800 bp and the rest were between 450-700 bp (Table 1). After identification of positive clones by colony-PCR, sequencing of these verified colonies with M13 primers revealed that four clones (approximately 25% of the clones) contained repetitive sequences.

Each sequence was compared with the nucleotide sequences available in national center for biotechnology information (NCBI) gen bank by BLAST. The number of 34, 24, 22 and 20 percent of the sequences were A, G, T and C after analyzing sequences, respectively. Four primer set designed for flanking the newly identified microsatellite loci (IBRC01 to IBRC04) were tested for their variability in 40 individuals (Bactrian camel cell lines). The microsatellite loci reported in this work have been deposited the genbank (NCBI). IBRC01 (OPA-03 RAPD primer) had a maximum repetitive unit with A (26) repeats and IBRC02 (IBRD RAPD primer) had a minimum repetitive unit with CTC (3) repeats.

All loci were polymorphic. Annealing temperature was 60 ˚C for IBRC01, 58 ˚C for IBRC02, IBRC03 and 57 ˚C for IBRC04. Three of four loci were significantly deviated from HWE, due to the heterozygote deficiency. No significant linkage disequilibrium was detected between the studied loci.

Expected size of alleles, observed heterozygosity (H_o) and expected heterozygosity (H_e) are shown in Table 1. Numbers of alleles per locus ranged from 3-5. The expected (H_e) and observed (H_o) heterozygosities ranged from 0.533 to 0.807 and 0.525 to 0.714, respectively (Table 2). Shannon Index is a parameter for determining the diversity index and ranged from 0.89 to 1.60. These deviations may have resulted from a small population size. Further investigation is needed for the understanding of the population genetics of Iranian Bactrian camel.

We selected sequences from bactrian camel, dromedary camel and alpaca available in NCBI, that they were similar to our sequences. Each sequence alignment with homologous sequences from bactrian, dromedary camel and alpaca. Alignment results were used to draw phylogenetic trees using the UPGMA method. All trees were divided into two branches.

The result showed that, except of OPA-03, OPB-05 and OPB-07 sequences, Iranian bactrian camel and bactrian camel sequences were more related to each other and Iranian bactrian camel and alpaca sequences had the highest genetic distance among all sequences. Iranian bactrian camel had the highest genetic distance with dromedary camel in OPA-03 sequences. In OPB-05 sequences, first branch consisted of the Iranian bactrian camel, dromedary camel and second branch consisted of the bactrian camel with alpaca. In OPB-07, bactrian camel and dromedary camel were in a clade and Iranian bactrian camel was consisted another clade (Figure 1a).

Finally, all sequences of each species were aligned in a row.

Table 2 Primer sequence, accession number (AN), repeat motif (RM), size range (SR), number of alleles (NA), observed heterozygosities (H_O), expected heterozygosities (H_E), Hardy–Weinberg equilibrium (HWE) and Shannon index (SI) for four microsatellite loci of Iranian *Camelus bactrianus*

Locus	Primer sequence (5' to 3')	AN	RM	SR (bp)	NA	Ho	H_E	HWE	SI
IBRC01	F: GATGGACCTGGAGATCGTCA R: GTAGTTCATCCGTGTCCCCT	KX237506	$(A)_{26}$	130-142	4	0.657	0.651	0.212	1.07
IBRC02	F:AGGGGACACTCATCCATCCA R: TGCATAAGCAGGGAAGGTGG	KX237507	$(CTC)_4$	220-228	3	0.66	0.533	0.032	0.89
IBRC03	F: GGGTCAGATAGACCAGGGGT R: GCCTAAGGGCTGGTTTGACT	KX237508	$(CT)_7$	280-290	5	0.525	0.807	0.000	1.60
IBRC04	F: GCTGTCTTCAGTGTCAGTATCC R: GTAGTTCATCCGTGTCCCCT	KU240014	$(GT)_{11}$	140-155	5	0.714	0.665	0.002	1.19

Alignment results were analyzed to construct phylogenetic trees using the UPGMA and N.J. methods. Results showed that first cluster were consisted Iranian bactrian camel and bactrian camel in a clade and dromedary camel in other clade and alpaca was consisted in the second cluster. So, Iranian bactrian camel and bactrian camel sequences were more related to each other (Figure 1b).

In the current study, we used bactrian camel fibroblast cell line. Using these cell lines has advantages such as availability, identification, lack of need for long-distance travel, no need to sampling, animal welfare attention and the availability of sufficient quantity and infinite bactrian camels DNA. Therefore, the use of these cell lines, causes centralization and oriented of the bactrian camel studies and to be an alternative technology for conservation genetic resources and prevent this breed from extinction (Daneshvar Amoli *et al.* 2017).

The results of PIMA method shown, about 20 percent of positive clones had repetitive sequences. These sequences were suitable for microsatellites loci. Previous studies have shown that this method compared to traditional methods for microsatellites isolation and identification has much better performance. In traditional methods, on average, two percent of the clones have repetitive sequences (Hatanaka, 2002). Sequences of A, GT, AG and AC had the highest repetitive and frequency. In general, observed heterozygosity was high at all loci. These data are in agreement with previous studies (Shahkarami *et al.* 2012; Toth *et al.* 2000).

Nei and Takezaki (1996) suggested that average heterozygosity must be between 0.3 and 0.8 in a population, in order to be a useful marker for measuring genetic variation. Our results for mean heterozygosity were within this range; therefore the identified markers in this study are suitable for studying genetic variation in Bactrian camel as well as in other species of camelidae family.

In current study, we characterized four new polymorphic loci for Iranian Bactrian camel using PIMA method. There are several procedures for isolation of microsatellite loci, among those; PIMA seems to have some advantages over other techniques which makes it more suitable for isolation of microsatellite loci in this kind of studies.

First, the PIMA is significantly cheaper in a laboratory which is not regularly performing hybridization techniques. Second, it has the ability to isolate both flanking regions simultaneously and third, PIMA avoids handling of radioactivity. Further, it utilizes the reagents, experience and equipment which are needed for PCR analysis. According to the results of this study, PIMA seems to have similar efficiency as reported in other species such as fishes (Chung-Jian *et al.* 2007; Lin *et al.* 2008; Sadder *et al.* 2015; Sanches and Galetti Jr, 2006; Zane *et al.* 2000).

Previous studies indicated four microsatellite loci (LCA68, VOLP59, LCA90 and GLM6), which were significantly associated with the breeding values for the fiber diameter trait in camel. Association between microsatellites marker and QTL in camel represented importance of microsatellites. Thus, the marker assisted selection could increase the efficiency and effectiveness of breeding and conservation programs (Paredes *et al.* 2014). In the current study, the mean value of observed heterozygosities was 0.639 and lower than the camel population in Sudan. Expected heterozygosities mean value was 0.664 and mean value of Shannon index was 1.19. All of them were lower than Sudan camel population (Eltanany *et al.* 2015). In another study, genetic diversity of the Iranian bactrian camel was analyzed by 16 microsatellites marker. The overall mean value of alleles and heterozygosities were 3.5 and 0.476, respectively (Shahkarami *et al.* 2012). These results are lower than our study results. So, these four new microsatellite loci are better than other loci for Iranian Bactrian camel future studies. We analyzed the genetic distances among species based on DNA sequences. The sequence data revealed that the smallest distance is between Iranian bactrian camel and bactrian camel and the largest distances are between Iranian bactrian camel and alpaca. The highest geographical distance between bactrian camel and alpaca corresponds to the highest genetic distance. The phylogenetic analysis indicated that breeds and species were grouped according to their geographic locations except a few sequences. A similar observation of population clustering according to their geographic origin has been reported in cattle and goat (Rout *et al.* 2008).

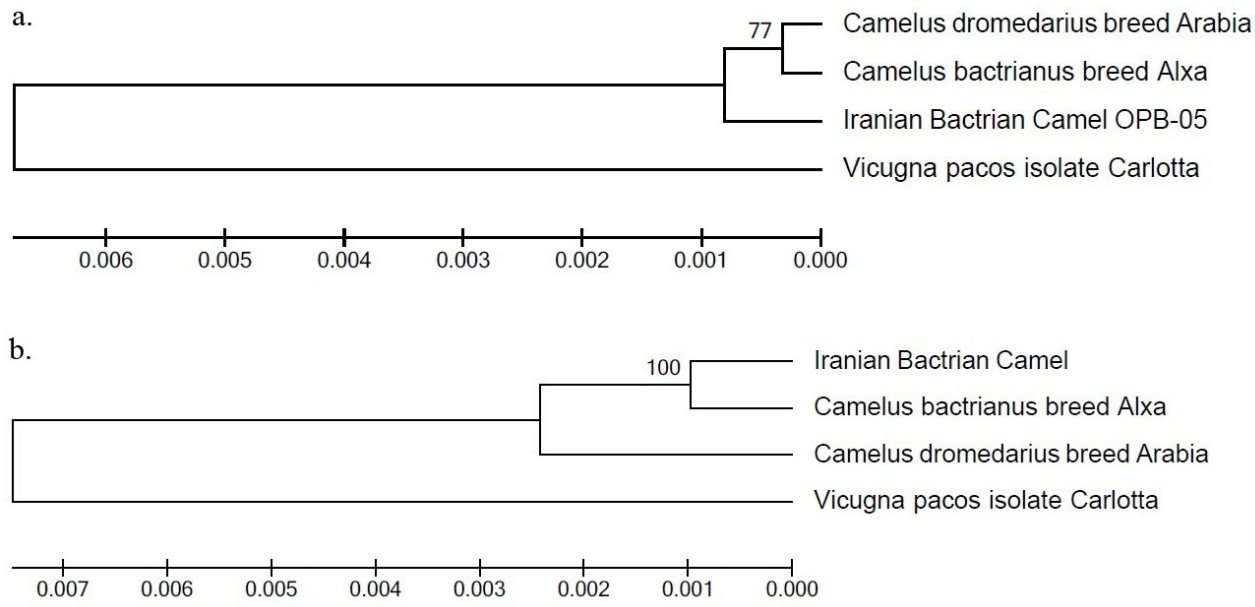

Figure 1 Evolutionary relationships of camelidae using UPGMA tree based on DNA sequences from bactrian camel, dromedary camel, alpaca (available in NCBI) and Iranian bactrian camel numbers at the nodes are bootstrap values from 1000 replicates
a. Phylogeny tree was constructed using OPB-05 sequences
b. Phylogeny tree was constructed using all 17 fragments sequences

This shows that geographically adjacent populations are more genetically related. The principal component analysis supported the grouping of animals and the distance between the breeds was significant.

CONCLUSION

In conclusion, we report, these four polymorphic microsatellite loci presented in this study were the first set of microsatellites marker designed specifically for Iranian *Camelus bactrianus*. These microsatellite markers provide the groundwork for further studies on the genetic structure, localizing loci associated with productive traits, gene flow, conservation management, molecular evolution of this vulnerable species. So, these markers used for parentage testing and identification bactrian camel cell lines.

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