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Full Length Research Paper

A novel male-specific DNA sequence for river buffaloes

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In this paper, we report identification of a novel male-specific DNA marker in Iranian river buffalo presenting the possibility of river buffalo gender determination using Random amplified polymorphic DNA-PCR (RAPD-PCR). Twenty eight random primers used for RAPD fingerprinting in order to isolation and characterization of microsatellite sequences using PIMA (PCR- based isolation of microsatellite array). All sharp bands related to different primers were isolated from the gel and inserted to a cloning vector and transformed to competent cells for nucleotide sequencing. Sequences were used as queries for BLAST tool. BLAST results showed that a sequence had high similarity with a part sequence of cattle Y chromosome sequences deposited in GenBank. A pair of primers (IRBMS-F and -R) was designed based on the sequence for amplifying the male-specific band by polymerase chain reaction (PCR) for Iranian river buffalo gender detection. Gender-specific bands in the gel were observed in all males but not in females. This showed that the gender of Iranian river buffalo could be easily and effectively identified by PCR technique using these primers.

Key words: Random amplified polymorphic DNA-PCR, gender detection, river buffaloes, male specific DNA marker.

INTRODUCTION

Iranian river buffaloes are used for many purposes including milk yield, meat production and draft power. Demands for beef have encouraged many farmers to raise them for slaughter. Iran has many areas that buffalo has potential superiority over cattle to reared there (Shokrollahi, 2010) . Gender is a genetic phenomenon, which assures the continuous generation of new gene combinations as substrate for evolution and genetic improvement through breeding. The livestock industry has been expected to be able to control the gender of domestic animals reproduction for a long time. Selection of offspring gender has tremendous impact on economics of many buffalo holders (Appa and Totey, 1999). There are many procedures for gender identification using DNA analysis including southern blot, dot blot. Several DNA probes has been used for gender determination using southern blot, however, this technique requires high amounts of undegraded DNA (Horng et al., 2004). Another technique for gender identification is polymerase chain reaction (PCR). PCR delivers rapid and liable results whenever small amounts of templates are available (Halser et al., 2002). Random amplified polymorphic DNA-PCR (RAPD-PCR) is based on amplification of DNA in PCR by oligonucleotide random primers. RAPD -PCR has been successfully applied in genetic studies of animal species identification as well as for gender determination of various animals. It is easy to perform, involves low cost, does not require known prior sequence of template and requires only a small amount of template DNA (Yen et al., 2001; Saifi et al., 2004). RAPD -PCR was employed for gender determination in plants (Banerjee et al., 1999; Shirkot et al., 2002; Deputy et al., 2002) and animal species such as cattle (Antoniou et al., 1995; Horng and Huang, 2000), Sheep (Gutierrez et al., 1997), pig (Horng and Huang, 2003), Pigeon (Huang et al., 2006), Geese (Huang et al., 2003) . In this paper we describe a novel male-specific DNA marker cloned from Iranian river buffaloes by RAPD-PCR.

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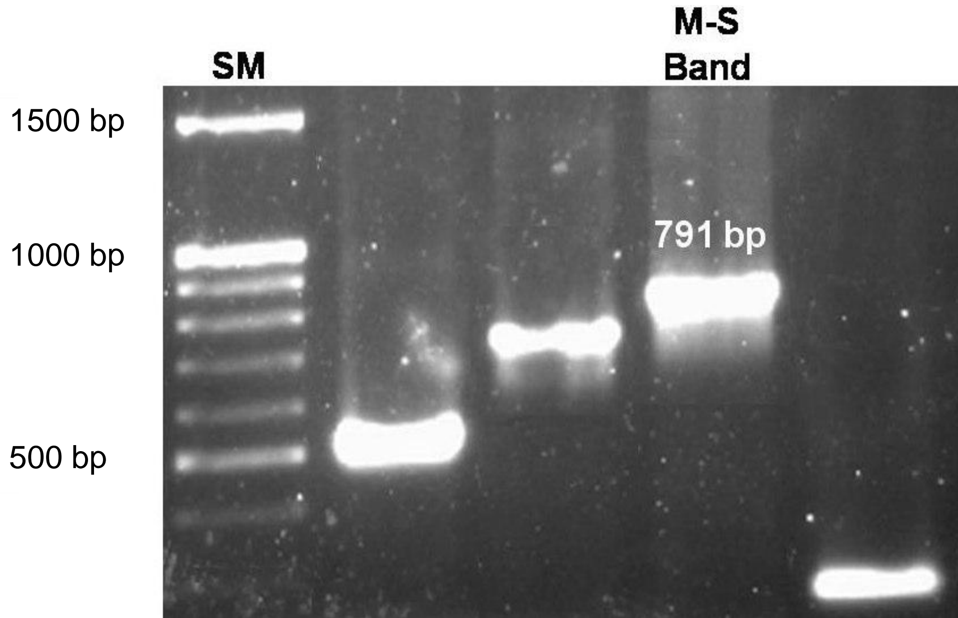

Figure 1. Purified RAPD Primer RP1 (5TAGCGTCGAC3) amplified from the genomic DNA of Iranian river buffalo. SM is ladder markers. M-S band shows male-specific band.

MATERIALS AND METHODS

DNA extraction

Five millilitre of blood was collected with anticoagulant from 80 male and female river buffaloes of Khuzestan, Azarbayejan, Guilan and Mazandaran provinces. The genomic DNA was prepared from blood using salting out procedure (Miller et al., 1988). The DNA was diluted with double distilled water to 50 ng/µl for performing RAPD-PCR.

RAPD-PCR

Twenty eight 10-base primers were used for PCR. The RAPD-PCR reactions carried out in a volume of 25 l using 1 l of template DNA (approximately 50 ng), 1 unit of Taq polymerase (Metabion, Germany) with reaction buffer (1X), 1.5 mM $MgCl_2$, 200 M each dNTP and 10 pmol of each RAPD primer. Amplification was performed using Eppendorf thermocycler with 94°C for 5 min followed by 45 cycles of 94°C for 30 s, 37.5°C for one min, 72°C for one min and finally extra extension at 72°C for 5 min. Amplificons were separated on 1% agarose gel. Gels were scanned under UV by photo-print gel documentation system. Sharp bands were selected and the related RAPD primers were amplified in large amount. PCR products were run on 0.8% agarose gel and stained by ethidium bromide. Interested bonds were recovered using QIA quick gel extraction kit (Qiagen INC., CA, USA).

Cloning of bands of interest

The purified bands were ligated into pDrive cloning vector with T4 DNA ligase at 4°C overnight and heat shock transformation with competent TOP10 cells was carried out at 42°C for 90 s and the cells were plated on LB-agar plates, which contained IPTG, X-Gal and ampicillin to allow blue and white colony selection.

Sequencing and sequence analysis

The inserted DNA fragments were sequenced in an ABI DNA sequencer using ABI prism dye terminator cycle sequencing reaction kits according to manufacturer's instruction. Sequences were verified by alignment using the BioEdit software (version 7.0, 9.0) to remove any redundant plasmids. The sequences were used as queries for database searching using BLAST tool. Sequence analysis and alignment were carried out using NCBI-BLASTN. Primer design was carried out using GENERUNNER software. PCR was performed using designed primers in normal condition.

RESULTS

Twenty eight RAPD primers were used to isolation and characterization of microsatellite sequences in Iranian river buffalo using PIMA (PCR- based isolation of microsatellite array) (Shokrollahi et al., 2009). One of these primers (RP1 (5TAGCGTCGAC3)) yielded four sharp bands. We cloned and sequenced all fragments of RP1 (Figure 1). Obtained sequences used as queries for BLAST tool. Alignment results showed that a fragment related to RP1 had high similarity with a part sequence of Y chromosome deposited sequences in GenBank (Figure 2). By taking high similarity of the sequence with sequences related to Y chromosome in cattle into account, a pair of primers IRBMS-F (5'-TCAAGTGCAGCATTTCTTGG-3') and -R (5'-TACCGCAAGGGGATATTCTG-3') was designed on the sequence of the interested fragment for amplifying a 317 bp male-specific sequence by PCR for Iranian river buffalo gender determination (Figure 3). Both male and female Iranian river buffalo genomic DNA were used as

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Query  11      GATTCCAATGCAATATATTGTTTAATCCTATAACTATTGTTAATACAGCTTTCTTTG-TT
               ||||||||||||||||||||||||| |||||||||| |||||||| | |||| |||| ||
Sbjct  107088  GATTCCAATGCAATATATTGTTTAAGCCTATAACTACTGTTAATATACCTTTATTTGTTT

Query  70      TAAATTTTTTAAACTTACCGCAAGGGGATATTCTGAC-AAAGCAGAAAAACATTTTGAGG
               ||  |||||| || |||| |||||| |||||| |||| ||||||||||| ||||||||||
Sbjct  107148  TATTTTTTTTTAA-TTACTGCAAGGAGATATTTTGACAAAAGCAGAAAAGCATTTTGAGG

Query  129     CATTGAAGGATACCTCCCTTCCTCTGCCCATTTGGTATCAAGATGGGTTGACTAAACAAT
                |||| |||| | ||||||||||||||||| |||||||     |  ||||||||||||||
Sbjct  107207  TATTG-AGGACATCTCCCTTCCTCTGCCCACTTGGTAT-----T--GTTGACTAAACAAT

Query  189     GGAAATCTGGAAAATTAATATTACAGGGAAAGGGG-TATGCTTGTATTTCTCCAGATCGA
               ||||||||||||||||||||||||||| |||  || ||||||||||| ||||||||| ||
Sbjct  107259  GGAAATCTGGAAAATTAATATTACAGGAAAAAAGGATATGCTTGTATATCTCCAGATGGA

Query  248     CCCAACGAAATCAGTTGGCTCCCTCTTCGGAAGATCCGCCCCAGAGGAGACCCCAG-CAT
                |||| ||||||| ||| ||||||||| ||||||||| |||||| |||| || | | |||
Sbjct  107319  TCCAATGAAATCAATTGTCTCCCTCTTTGGAAGATCCACCCCAGGGGAG-CCACTGACAT

Query  307     TCAAGATAGAGAAGAAATGGCAAAATCCCCAGGAGGAGGAGTTTCCAGTACAAGCCATGG
               ||||||||||||||||||||  |||||| ||| |||| || |||||| ||||||||||||
Sbjct  107378  TCAAGATAGAGAAGAAATGGTGAAATCCTCAGAAGGAAGAATTTCCAATACAAGCCATGG

Query  367     CAACTTTA-AAACTTTCCAAGAAATGCTGCACTTGAGGTCATCGACCTTATGATCTCCC-
               |  ||||| ||| ||||||||||||||  |||| ||  ||||| |  |||||||||||| 
Sbjct  107438  CGGCTTTACAAA-TTTCCAAGAAATGCCACACTCGATATCATCAAATTTATGATCTCCCT

Query  425     AACCTGGGGACAGATAAAAACCCTTACTAATCAAGCTG-AAT-TCT-------CAACAGG
               ||  ||| ||||||||||| || ||||| |||||| || ||  |||       |||||||
Sbjct  107497  AAT-TGGTGACAGATAAAA-CCTTTACTTATCAAG-TGGAAAATCTGGTTTTTCAACAGG

Query  476     CAATGCCTCAGAATCCTGAAAATATTTTTGTCGCTATGCTTGCTTTGCTTGCTTTTGCTT
                ||||| |||||| ||||| |||||||||||| |||||||||||||||||||||||||||
Sbjct  107554  GAATGCTTCAGAACCCTGAGAATATTTTTGTCACTATGCTTGCTTTGCTTGCTTTTGCTT

Query  536     CCCCTGCTCAGGCTGACTTGATTAATCACACTTATTAGCCTTATATATCTAAcccccccT
               ||||  || ||||| ||||||||||||||||||||| | ||||| || |||||||||| |
Sbjct  107614  CCCCCACTGAGGCTTACTTGATTAATCACACTTATTGGGCTTATGTAACTAACCCCCC-T

Query  596     TTATTGCAGGTTGTAGAATGGACAGATATAGGACCAGTTGAT-CC-ACTAATGAC-TCAG
               |||||||||||||||||||| |||||||||||||  ||  || || ||||||||| | ||
Sbjct  107673  TTATTGCAGGTTGTAGAATGTACAGATATAGGACTGGTC-ATACCCACTAATGACAT-AG

Query  653     TACATATGCCTCCTCCTTGGAGCTTGGAGGGGCCCTCTCATCCTGAGGAAGAAGGAAGAT
                ||||||||| ||||||||||| ||||| ||| || ||||||| ||||||||||||||| 
Sbjct  107731  CACATATGCCCCCTCCTTGGAGTTTGGAAGGGACCGCTCATCCGGAGGAAGAAGGAAGAC

Query  713     TGATTAACATTTCTCTAGGCTATAAAATCCTTCCTTTATGCATGGGCCCAGCAGA-TTAT
                 ||||||||||||||||| ||| || ||||||||||||| |||||||||||||| ||||
Sbjct  107791  AAATTAACATTTCTCTAGGTTATGAAGTCCTTCCTTTATGGATGGGCCCAGCAGAATTAT

Query  772     GTATTAATGTTAGT  785
               ||||||||||||||
Sbjct  107851  GTATTAATGTTAGT  107864
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Figure 2. Alignment of the sequence Iranian river buffalo (Query) with a part of *Bos taurus* Y Chromosome sequence (accession number: AC225773.3) (Sbjct). Vertical lines indicate the nucleotide identity according to the Iranian river buffalo sequence.

TCAAGTGCAGCATTTCTTGGAAAGTTTTAAAGTTGCCATGGCTTGTACTGGAAACTCCT
IRBMS-F →
CCTCCTGGGGATTTTGCCATTTCTTCTCTATCTTGAATGCTGGGGTCTCCTCTGGGGC
GGATCTTCCGAAGAGGGAGCCAACTGATTTCGTTGGGTCGATCTGGAGAAATACAAGC
ATACCCCTTTCCCTGTAATATTAATTTTCCAGATTTCCATTGTTTAGTCAACCCATCTTG
ATACCAAATGGGCAGAGGAAGGGAGGTATCCTTCAATGCCTCAAAATGTTTTTCTGCTT
TGT**CAGAATATCCCCTTGCGGTA**
← IRBMS-R

Figure 3. Male-specific DNA sequence of Iranian river buffalo. Two primers, IRBMS-F and -R, corresponding to segments at both ends were designed.

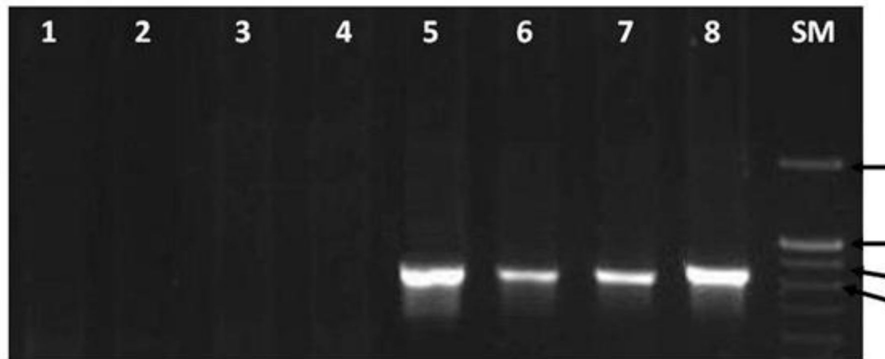

Figure 4. Electrophoretogram of PCR products amplified from the Iranian river buffalo genomic DNA with male- specific primer (IRBMS-F and -R). Male-specific band in length 317 bp were represented in the gel for only all males (lane 5-8). Lane 1-4 shows amplification of female genomic DNA with IRBMS-F and –R primers. SM is ladder markers.

templates and amplified with these two primers by PCR. By electrophoresis of PCR product and staining with ethidium bromide, male-specific band observed in the gel but not for females (Figure 4).

DISCUSSION

Gender usually forms the largest single division within a species and causes differences in physiology, behaviour and economic usefulness of different individuals of a species. Molecular sexing is an attractive option since it can potentially provide an accurate and rapid means for determination of gender, especially if based on non-invasive techniques (Fridolfsson et al., 1999). RAPD is a simple, cheap and does not need designed control for the PCR reaction. However, RAPD is known to be more sensitive with reaction condition (e.g., template quality, ramping speed and type of instrument used) than standard PCR when performed with longer and specific primers. In this study, the RAPD fingerprints from Iranian river buffalos amplified by RAPD-PCR with twenty eight primers to isolation of microsatellite sequences by PIMA. Four Sharp bands, amplified with RP1 primer, can be observed in the fingerprints of genomic DNA at 248 bp, 531 bp, 700 bp and 791 bp positions (Figure 1). DNA fragments cloned and sequenced related to available RAPD primers to obtain microsatellite sequences. The sequences of different fragments were analysed by BLAST tool to find similar sequences. Sequence analysis by BLAST tool revealed that a sequence had have similarity (84 to 87%) with some Y chromosome sequences deposited in GenBank and no buffalo reported DNA fragment have similarity to this fragment, so it can be used as a novel marker in river buffalo. Two male specific primers (IRBMS-F and -R) were designed based on the cloned 791 bp male-specific sequence in Iranian river buffalo. The single band of the male specific PCR fragment (317 bp) was generated using the designed primers and observed in only in the males but not in any females (Figure 3). These primers used to sex-type about 40 individuals in four populations of Iranian river buffalo (Azarbayejan, Guilan, Mazandaran and Khuzestan) and verified high accuracy.

The annealing temperature influences the specificity of the amplification reaction. If the temperature is too high, no annealing occurs at all, but if the temperature is too low, nonspecific annealing might increase dramatically.

Sometimes the evaluation of optimum annealing temperature is the most time-consuming part of an optimization strategy (Rolfs et al., 1992). In the present study, it is found that the most appropriate annealing temperatures were 37.5°C for RAPD-PCR and 52°C for PCR.

In conclusion, a novel male-specific Iranian river buffalo DNA sequence was obtained using cloning of a RAPD fragment. Two male-specific primers, IRBMS-F and -R could be accurately and rapidly used for river buffalo gender determination by PCR.

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