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Allele Specific and tetra-primer ARMS PCR Assays for Rapid Screening of SNPs at Male-specific Region of Y-chromosome in Cattle

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ABSTRACT: *Markers on the male-specific region of Y- chromosome (MSY) in mammals, paternally inherited in a haploid way, have widely been used to study the origin of species, range expansion, admixture of populations and migration in animals. Molecular variation in the Y chromosome provides information about genetic diversity, since it reveals the distribution of paternal lineages. Recent discovery of five polymorphic sites on cattle Y-chromosome genes (DDX3Y, UTY and ZFY) allows identifying three haplotypes (Y1, Y2 and Y3) in cattle. Here we report the optimization and validation of allele specific PCR and tetra-primer ARMS PCR protocol for screening three SNPs on genes (DDX3Y -intron 1, 425>C/T; ZFY -intron 9, 120> C/T; and ZFY- intron 10, 655> C/T) located at MSY of bull Y-chromosome. Although, both the techniques are found to be efficient in exploring Y-SNPs variation; however we propose to utilize allele specific PCR over and tetra-primer ARMS PCR for screening SNPs at MSY of Y- chromosome in cattle as it requires little standardization and thereby less time consuming. The standardized protocols may be useful for large scale screening of those Y-SNPs and analysis of Y-haplotypes in diverse native cattle breeds, exotic and crossbred cattle populations with high accuracy and reliability.*

Keywords: *Single nucleotide polymorphism, Amplification Refractory Mutation System (ARMS), AS-PCR, Crossbred (Bos taurus X Bos indicus)*

INTRODUCTION

In livestock species, Y chromosome studies are of particular interest as because in common breeding strategies only a few males contribute genetically to the next generation (Lindgren *et al.*, 2004). The mammalian Y is gene poor, male specific chromosome often determining sex in a dominant fashion and is inherited clonally from father to son. Y chromosome analyses may thus complement studies using mitochondrial DNA for inferring sex-specific population genetic processes. The mammalian Y chromosome has two components, a pseudoautosomal region (PAR) which frequently recombines with the X chromosome and a non-recombining region. This non-recombining region is called the male-specific region of the Y chromosome, the MSY, and comprises 95% of the length of the chromosome in mammals. Remaining 5% that is genetically similar to X constitues the PAR, in the telomere ends of the Y chromosome and recombine with the X chromosome during meiosis.

Markers on the MSY, paternally inherited in a haploid way, have widely been used for studying the origin of species, range expansion, admixture of populations, and migration in animals (Pidancier et al., 2006). Molecular variation in the Y chromosome provides information about genetic diversity, since it reveals the pattern of distribution of paternal lineages.

The mammalian Y chromosome had been poorly characterized compared with that of the X chromosome and autosomes because of the sequencing difficulties imposed by the abundance of repetitive sequences, inherent tendency of Y chromosome's genes to degenerate during evolution resulting poor gene content with limited transcriptional potential (Quintana-Murci and Fellous 2001). Using recently available technologies, it has been reported that Y chromosome of bulls contains more active genes than the Y chromosome of other mammals (Chang *et al.*, 2013). This discovery may help biologists to understand the evolution of cattle and other species in a greater depth and to help breeders and farmers more effectively maintain genetic diversity and increase their livestock's

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fertility. Moreover, with the advent of sequencing technology a lot more sequencing information will be available in near future leading to identification of numerous Y-specific variations. Götherström *et al.* (2005) identified five polymorphic sites on the cattle Y-chromosome genes (DDX3Y, UTY and ZFY), allowing identification of three haplotypes (Y1, Y2 and Y3) in cattle. Majority of the previous studies conducted to explore Y-chromosome SNPs and haplotype diversity of different mammalian species are mainly sequence based. The present work aimed at developing alternative genotyping protocols (Allele specific PCR and/or tetra-primer ARMS PCR) for genotyping Y chromosome variations.

MATERIALS AND METHODS

Sample collection

Blood samples from Sahiwal, Tharparkar *(Bos indicus*) and HF crossbred (*Bos taurus* X *Bos indicus*) bulls were collected from the jugular vein into EDTA containing vacutainer tubes. Genomic DNA was isolated and puriûed from the blood cells using the standard phenol–chloroform–isoamyl alcohol extraction followed by ethanol precipitation (Sambrook and Russell, 2001). The quality and quantity of isolated DNA were determined using agarose gel electrophoresis (0.8%) and spectrophotometer (NanoDrop, GE Healthcare).Good quality DNA having OD 260/280 ratio between 1.8 and 2 were used for further analysis.

Primer designing

Allele Specific Primers and Tetra-primer ARMS-PCR primers were designed using online software BatchPrimer3 v1.0 (*http://probes.pw.usda.gov/batchprimer3*). The genomic specificity of the primers was tested using the Primer-BLAST program (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/*). Details of primers used in the present investigation are listed in Table 1 and 2.

Table 1
Allele specific PCR primers detail for screening three SNPs on bull Y- chromosomes

SNP	*Position*	*Primer ID*	*Primer Sequence (5′-3′)*	*Conc. (μM)*	*Ta (°C)*	*Product size (bp)*
DDX3Y-1	Intron 1425>C/T AY928816	**AS-**DDX3Y1-(T)	CAAATATTTCTTGACATTAATGTG**AT**	1.0	52	182
		AS-DDX3Y1-(C)	CAAATATTTCTTGACATTAATGTG**AC**	1.0		
		DDX3Y1-CR	TCCAAAAACATAGCTGTATATTAGTAAT	1.0		
ZFY9	Intron 9120> C/T AY928828	**AS-**ZFY-9 (T)	TACTAATGAACTGATTTAAGTAAAAA**ACA**	1.0	52	87
		AS-ZFY-9 (C)	TACTAATGAACTGATTTAAGTAAAAA**ACG**	1.0		
		ZFY9-CR	CATAAAGAAAGTTCCTATTAAAGTTAAA	1.0		
ZFY10	Intron 10655> C/T AF241271	**AS-**ZFY-10(C)	TTTAATTATATTTTAATTGGTACAGTCC**TC**	1.0	52	374
		AS-ZFY-10(T)	TTTAATTATATTTTAATTGGTACAGTCC**TT**	1.0		
		ZFY10-CR	CTTCTTATTGGTAGTGTAATCACAATCA	1.0		

Table 2
Detail of primers for tetra-primer ARMS PCR to screen three SNPs on bull Y- chromosome

SNP	*Position*	*Primer ID*	*Primer Sequence (5′-3′)*	*Conc. (μM)*	*Ta* (°C)*	*Product size (bp)*
DDX3Y-1	Intron 1425>C/T AY928816	DDX3Y1-FIN (T)	CAAATATTTCTTGACATTAATGTG**AT**	1.0	50/48	182
		DDX3Y1-RIN (C)	ATTTATACATATTTAATTGAAAAAG**TCG**	1.0		131
		DDX3Y1-FO	CTGTTTAACAGGAATTAAAAGTAGTAAG	1.0		260
		DDX3Y1-RO	TCCAAAAACATAGCTGTATATTAGTAAT	1.0		
ZFY10	Intron 10655> C/T AF241271	ZFY10-FIN (C)	TTTAATTATATTTTAATTGGTACAGTCC**TC**	1.0	54/50	374
		ZFY10-RIN(T)	AGTGAATTTGGTCCAGAAAACTC**CTA**	1.0		415
		ZFY10-FO	GAAAACTATCAGGTTACATTATTGGAAA	1.0		733
		ZFY10-RO	CTTCTTATTGGTAGTGTAATCACAATCA	1.0		

*Ta: Annealing temperature; * Table 4 for detail

Polymerase chain reaction

Allele specific PCR

In allele specific PCR, for each sample, two PCR reactions were run in parallel, one with primer having C at 3′ end and second reaction with primer with having T at 3′ end. The reverse primer was common for both the reaction. To increase specificity, an extra mismatch is also intentionally introduced at the third position from the 3' end of each of the two allele specific primers. Final reaction mix contained 1X PCR Buffer, 1.5 mM MgCl2, 1.00 U of Taq DNA Polymerase (Sigma Aldrich), 200 ìM dNTPs (Sigma Aldrich), 10 pM of each primer and 50 ng of genomic DNA in a ûnal volume of 25µL. Ampliûcation was performed on a PTC-200 thermal cycler (MJ research, Inc) with specific cycling conditions as mentioned in table 3. The PCR products were analyzed by electrophoresis on 1.5% Tris-acetate-EDTA/ethidium bromide agarose gels, visualized under ultraviolet illumination and the presence or absence of specific band (Table 2) was indicative of presence or absence of a particular allele. A sample was considered negative for a particular allele when the amplicon was absent. Furthermore, template DNA is same for each allele-speciûc genotyping reaction serves as a control for the false positive or negative reaction secondary to extraction failure or the presence of an inhibitor.

Table 3
PCR programs for Allele Specific PCR

DDX3Y-1	*ZFY9*	*ZFY10*
95°C- 5 min	95°C- 5 min	95°C- 5 min
95°C-25s 52°C-15s 72°C-25s } 33cycles	95°C-25s 54°C-15s 72°C-25s } 30 cycles	95°C-25s 52°C-15s 72°C-25s } 30 cycles
72°C-10 min	72°C-10 min	72°C-10 min

Tetra-primers ARMS PCR

This procedure adopts principles of the tetra-primer PCR method and the amplification refractory mutation system (ARMS). Four primers are required to amplify a larger fragment from template DNA containing the SNP and two smaller fragments representing each of the two allele specific products. Primers are designed in such a way that the amplicons of two alleles differ in sizes and can be resolved by agarose gel electrophoresis. To enhance the specificity of the reaction, in addition to the first mismatch at the 3' end of allele specific primers, an extra mismatch is also deliberately introduced at the third position from the 3' end of each of the two inner allele specific primers (Table 2). PCR was carried out from a starting template of approximately 50 ng of genomic DNA in a final reaction volume of 25 ìl containing 1X Taq DNA polymerase buffer (Sigma), 1.5 mM MgCl (Sigma), 200 ìM dNTPs (Sigma), each primer as mentioned (table 2) and 1U Taq polymerase (Sigma). Ampliûcation was performed on a PTC-200 thermal cycler (MJ research, Inc) and the cycling conditions were as mentioned in table 4.

Table 4
PCR programs for tetra-primers ARMS PCR

DDX3Y-1	*ZFY9*	*ZFY10*
94°C- 3 min	95°C- 4min	95°C- 4 min
94°C-15s 50°C-20s 72°C-30s } 5 cycles	94°C-30s 54°C-20s 72°C-45s } 5 cycles	94°C-30s 54°C-20s 72°C-45s } 5 cycles
94°C-15s 48°C-20s 72°C-30s } 30 cycles	94°C-30s 50°C-20s 72°C-45s } 30 cycles	94°C-30s 50°C-20s 72°C-45s } 30 cycles
72°C-5 min	72°C-5 min	72°C-5 min

Validation of the assay

To rule out any false results arising from non-specific amplification of Allele specific PCR and/or tetra-primers ARMS PCR is very essential. To validate our present assays, specific primers were designed (Table 5) encompassing the sites of interest. Representative amplicons of 259, 286, 249 bp pertaining to DDX3Y-1, ZFY-9 and ZFY-10 genes were sequenced directly using automated DNA sequencer. Results obtained by Tetra-primer ARMS-PCR, Allele Specific PCR and direct sequencing were compared. In all the assays negative (no template) and a female DNA (Crossbred and *Bos indicus* cow) were used as control to rule out possible PCR contamination and nonspeciûc amplification.

RESULTS AND DISCUSSION

Due to exclusively paternal inheritance, Y-chromosome has been extensively used in evolutionary genetics. Variations of MSY regions are mostly transmitted as haplotype because of absence of crossing over. Moreover, in any animal either one of alternative form of a putative SNP may be available at a time. Therefore, heterozygous animals are not observed. Most of the studies of Y-SNP screening are sequencing based which are laborious, time and DNA consuming. We here tried to develop alternative approaches like Allele specific PCR and Tetra-primers ARMS PCR for screening SNPs at MSY of bull Y-chromosome. These techniques are economical as well as technically simple and can easily be applied to

Table 5
Primer details used for validation of tetra-primer ARMS PCR and Allele Specific PCR results

Locus	*Region*	*Size (bp)*	*Ta (°C)*	*Primer sequences (52 -32)*
ZFY-9	Intron 9	259	58	F—TCACATTGCAGCTTTAGGATTG R—CCTTCACTTGGCAGATGGAT
ZFY-10	Intron 10	286	57	F— CCAAAATGGTTGAGCTTTATGA R— GGAGCATAAGTGATCCAATGAA
DDX3Y-1	Intron 1	249	58	F—TGAACCACTAGGGAGGTCATC R—TTCCAATTTAGCTGTGGTTATCTG

generate large data set so as to draw genetic relationship across breeds as well as to reconstitute cattle demographic history.

In the present investigation, three Y-specific cattle SNPs viz., DDX3Y-1 (425>C/T), ZFY-9 (120> C/T) and ZFY-10 (655> C/T) screening protocols were developed using both allele specific PCR (Fig.1) and tetra-primers ARMS PCR (Fig. 2) methods.

The allelic pattern obtained from alternative protocols (allele specific PCR and tetra-primers ARMS

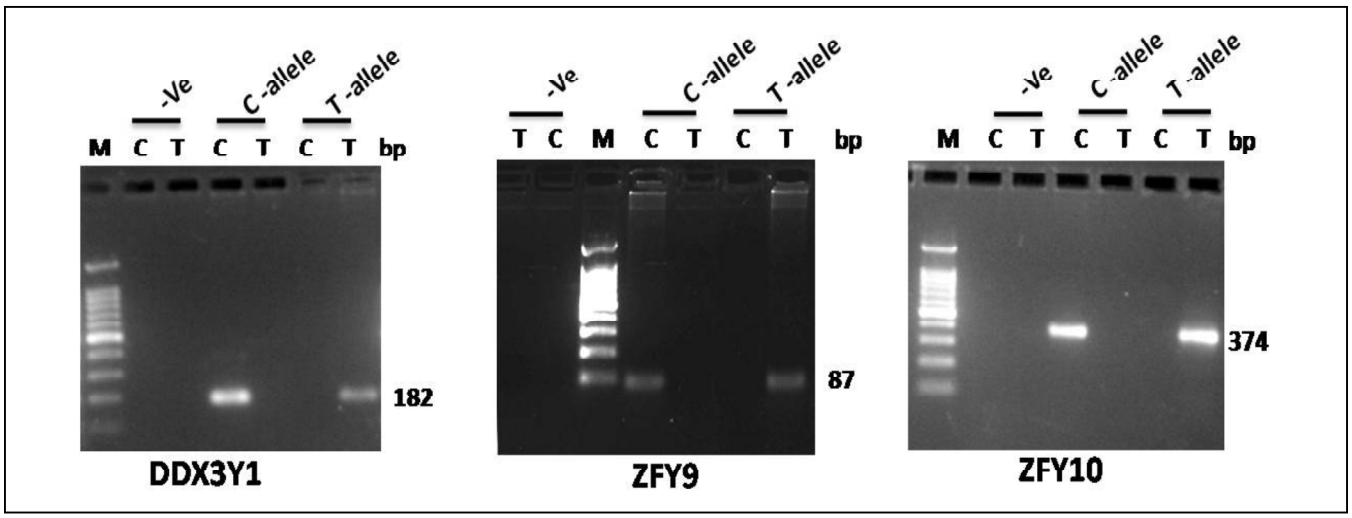

Figure 1: Screening of Y- chromosomal variation through allele specific PCR

M- Molecular DNA marker of 100 bp. In all the three regions C allele observed in HF crossbred bulls whereas T allele in Bos indicus bull samples.

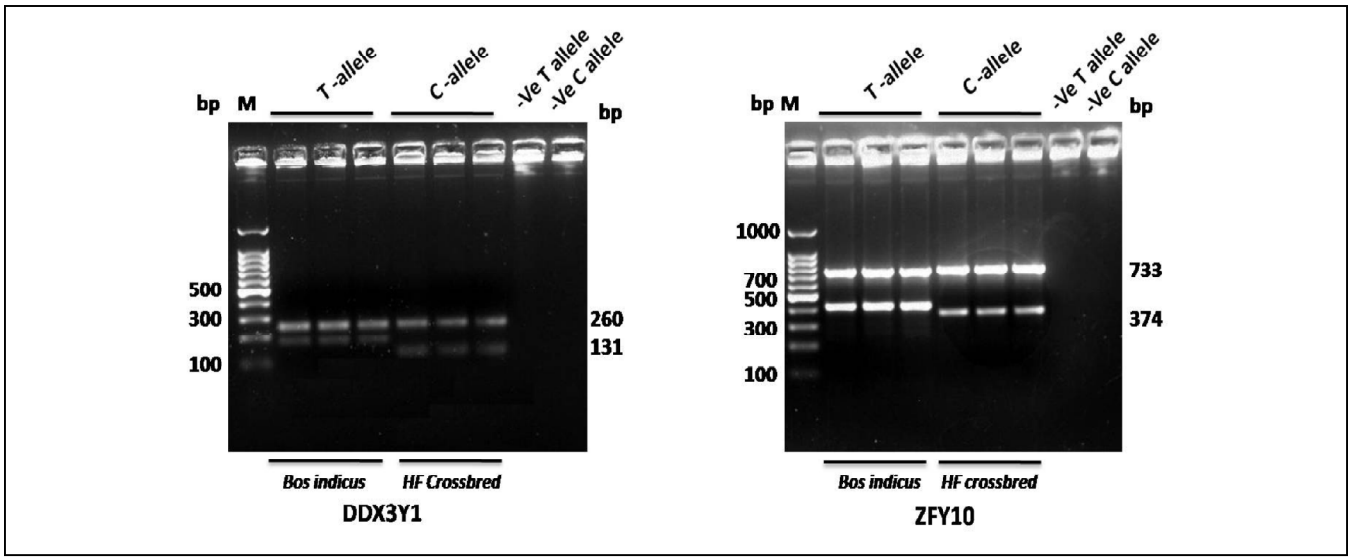

Figure 2: Screening of Y- chromosomal variations through tetra-primers ARMS PCR

PCR) was further confirmed by direct nucleotide sequencing. Absolute concordance was observed between results obtained through direct sequencing and Allele specific-PCR & tetra-primers ARMS PCR. Moreover, in every assay one negative (no template) and a female DNA used as control revealed no template contamination and nonspeciûc ampliûcation.

While using such types of PCR genotyping assays, one has to remember that mismatches extend at a lower rate, resulting in an amplification delay. Therefore, careful optimization of number of PCR cycles in each assay (Table 3) need to be standardized as excess number of cycles could result in poor genotyping discrimination.

These standardized and cost effective protocols may now be useful for large scale screening of Y-SNPs (DDX3Y1- 425>C/T; ZFY9- 120> C/T and ZFY10- 655> C/T) in diverse native cattle breeds, exotic and crossbred cattle populations with high accuracy and reliability. Although, both the techniques are useful in Y-SNP screening; however we propose to utilize allele specific PCR over tetra-primer ARMS PCR for screening SNPs at MSY of Y- chromosome in cattle as they require little standardization and thereby less time consuming.

CONCLUSION

Here we report the optimization and validation of Allele specific PCR and tetra-primer ARMS PCR protocol for screening three SNPs (DDX3Y1- 425>C/T; ZFY9- 120> C/T and ZFY10- 655> C/T) on MSY of bull Y-chromosome. These specific and reliable protocols may be useful for screening those Y-SNPs in diverse native cattle breeds, exotic and crossbred cattle. Base on present investigation, we propose that allele specific PCR will be better than tetra-primer ARMS PCR for screening of SNPs at male-specific region of Y- chromosome (MSY) in cattle as they are standardized without much effort. These genotyping protocols may be useful in large scale future SNP genotyping and analysis of Y-haplotypes across the cattle population. Further, the assay can be optimize for SNPlexing (single assay system) i.e., detection of all the three SNPs in one reaction and hence making it more economical, easy, time and DNA saving.

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