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# Y-chromosome variation in Indian native cattle breeds and crossbred population

**Suchit Kumar[1], Indrajit Ganguly*, Sanjeev Singh, Monika Sodhi and Mukesh Bhakat[1]**

ICAR-National Bureau of Animal Genetic Resources,
Karnal, Haryana-132 001, India.
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## ABSTRACT

The paternally inherited Y chromosome markers have been used widely in population genetic studies to trace paternal lineages, to understand differences in migration pattern and populations admixture in animals. In the absence of crossing over, Y-chromosomal markers in the non-recombining male-specific region (MSY) are mostly transmitted as a haplotype. Recent studies of five polymorphic sites on DDX3Y, UTY and ZFY genes of bull MSY assisted in the identification of three haplogroups (Y1, Y2 and Y3) in contemporary cattle. Here we report the screening of five SNPs (ZFY9- 120> C/T; ZFY10- 655> C/T; DDX3Y1- 425>C/T; DDX3Y7 -123>C/T and UTY19-423>C/A) of bull MSY employing optimized and validated allele-specific PCR (AS-PCR) protocols that are useful in effective differentiation of bull/semen samples of *Bos indicus* and *Bos taurus* origin. Three haplogroups (Y1, Y2 and Y3) were identified in the present study by the screening of 181 bulls from 10 native cattle breeds and 50 HF crossbred. Y1 and Y2 haplogroups were restricted to HF crossbred with a frequency of 0.98 and 0.02, respectively. The high frequency of Y1 haplogroups is possibly due to the occurrence of Y1 lineage predominantly in HF bulls. All the native cattle breeds were observed to have pure indicine lineage (Y3). These cost effective AS-PCR protocols may be useful for reliable and accurate genotyping of Y-SNPs in diverse native cattle breeds, exotic and crossbred cattle populations.

**Key words:** Diversity, MSY, Pseudoautosomal region, Y-Haplotype, Y-SNPs, Zebu cattle.

## INTRODUCTION

Studies of male lineages contribute towards better understanding of the origin and relationships among domestic breeds (Edwards *et al*., 2000; Lindgren *et al*., 2004; Gotherstrom *et al*., 2005; Anderung *et al*., 2005; Li *et al*., 2007). Microsatellites (STRs) mapped to the non-recombining region (Vaiman *et al*., 1994; Kappes *et al*., 1997; Liu *et al*., 2003) of Y-chromosome have been employed to study the events of domestication and differentiation among bovid species. Some of these markers have also been used to detect introgression and to distinguish between *Bos taurus* and *Bos indicus* patrilines (Edwards *et al*., 2000; Hanotte *et al*., 2000; Giovambattista *et al*., 2000; Li *et al*., 2007). Genetic studies pertaining to cattle Y-chromosomes are relatively less (Verkaar *et al*., 2004) and have mainly focused on the assessment of male migration patterns and admixture of *B. taurus* and *B. indicus* (Hanotte *et al*., 2000; Anderung *et al*., 2007; Edwards *et al*., 2007) or the assessment of differences in diversity among different breeds (Ginja *et al.,* 2009; Kantanen *et al.,* 2009). Recent discovery of five polymorphic sites on the cattle Y-chromosome (Gotherstrom *et al*., 2005) resulted in the identification of three haplogroups (Y1, Y2 and Y3) in contemporary cattle. European cattle breeds carry two haplogroups (Y1 and Y2), with Y1 being more frequent in *B. taurus* of north-western Europe, Y2 being predominant in *B. taurus* of southern Europe and Anatolian cattle. The Y3 was observed exclusively in *B. indicus*. In India, research reports on cattle Y-chromosome are scanty. The lineages and diversity based on Y-chromosome markers has not yet been extensively analyzed in Indian native cattle breeds and crossbred population. Therefore, the present investigation was undertaken to screen Y-chromosome specific variations in Indian native cattle breeds and crossbred population and to explore the existing paternal lineages/Y-haplogroups.

## MATERIALS AND METHODS

**Sample collection and DNA extraction:** Blood samples (n=181) were collected randomly from 10 native Indian breeds and HF crossbred bulls: Gir (15), Kankrej (8), Khillar (15), Mewati (15), Nagori (15), Nimari (14), Rathi (15), Sahiwal (10), Tharparkar (10), Malnad Gidda (14) and HF x zebu crossbred (50). The native cattle breeds covering different geographical region of India as well as having different utility purposes (*viz*., milch, dual and draught) were included (Fig. 1). Additionally, two Tharparkar and two HF crossbred cows were also included as control. Genomic DNA was extracted from blood samples using phenol-chloroform extraction method (Sambrook and Russell, 2001).

*Corresponding author's e-mail: drindrajit@gmail.com
[1]DCB Division, ICAR-National Dairy Research Institute, Karnal-132001, Haryana, India.

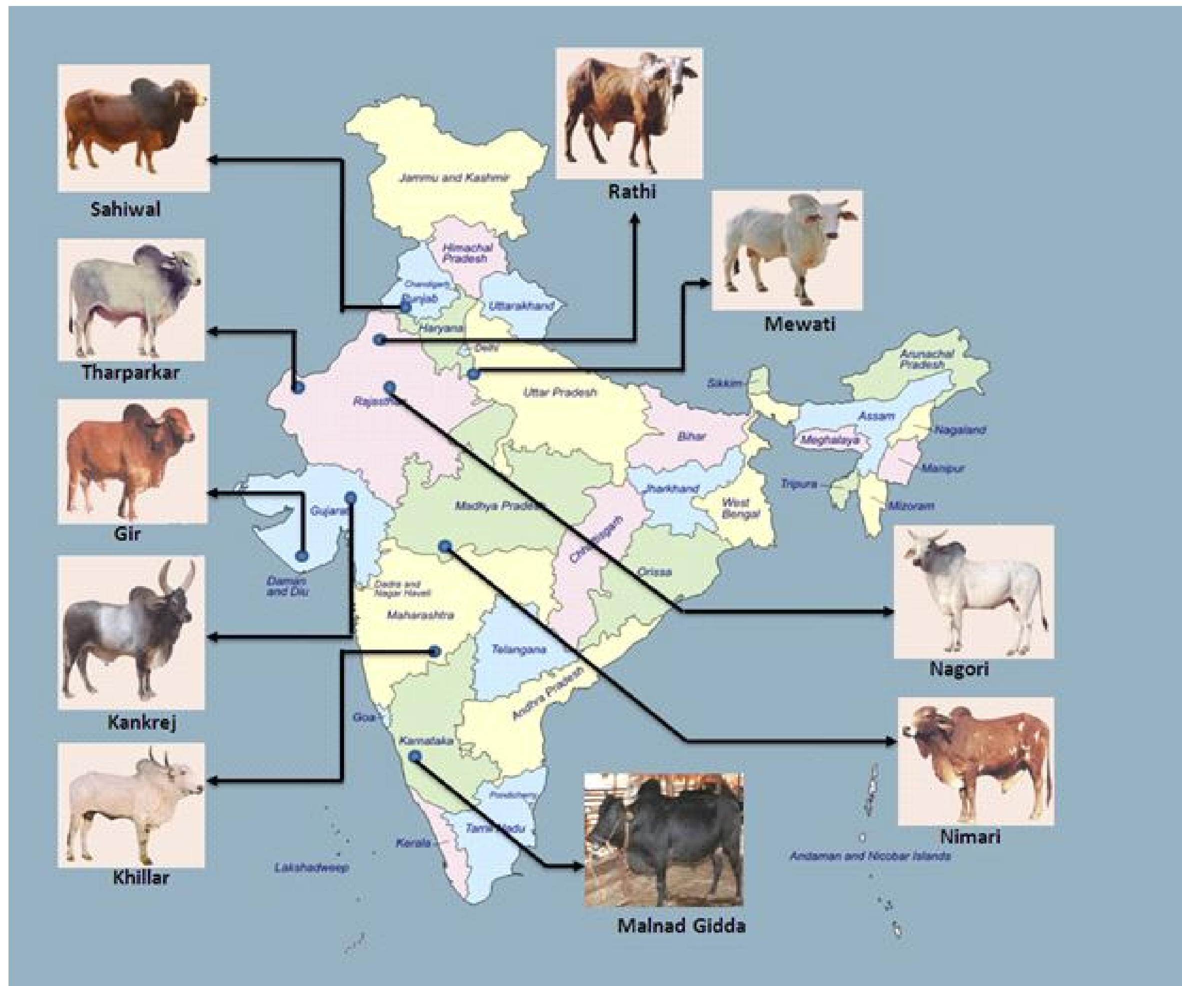


**Fig 1:** Indian native cattle breeds included in the present study

**PCR amplification of targeted regions of bovine Y specific genes:** Regions surrounding five Y-specific SNP markers (ZFY-9, ZFY-10, DDX3Y-1, DDX3Y-7 and UTY-19) were amplified (Table 1) as described (Gotherstrom *et al*., 2005; Ginja *et al*., 2009). The reaction mix contained 1X PCR Buffer, 1.5 mM $MgCl_2$, 1.00 U of Taq DNA Polymerase (Sigma Aldrich), 200 µM dNTPs (Sigma Aldrich), 10 pmole of each primer (Sigma Aldrich) and 100 ng of genomic DNA in a final volume of 25µl. Ampliûcation was performed on a PTC-200 thermal cycler (MJ research, Inc). In order to check any PCR carryover, negative control without template (genomic DNA) was always included. Amplification cycle comprised of denaturation at $95^0$ C for 5 min, followed by 35 cycles at $95^0$C for 30s, 53–$58^0$C for 20s, $72^0$C for 30s, and a final extension step at $72^0$C for 5 min. The PCR products were electrophoresed through 1.0% agarose gel and amplicons were visualized under UV transillumination (UVP Bioimaging Systems, Upland, CA®).

**PCR Product purification and sequencing:** The amplified PCR products from three representative samples of Sahiwal and HF crossbred bulls were purified, sequenced and sequence information was further used as a reference for designing and developing genotyping protocol.

**Designing of primers for developing genotyping protocol:** Allele Specific PCR (AS-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 Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) after adjusting the base to normal at third position from the 3' end of allele specific primers. Here we standardized AS-PCR for the genotyping of DDX3Y-7:123>C/T and UTY-19:423>C/A. Other three SNPs (*viz*., ZFY-9:120>C/T; ZFY-10:655>C/T and DDX3Y-1:425>C/T) were screened as per standardized AS-PCR methods described (Kumar *et al*., 2015). In the AS-PCR of ZFY9, ZFY10, DDX3Y1 and DDX3Y7, for each sample, two PCR reactions were run in parallel, one reaction with primer to recognize C at 3’ end and second reaction with primer to distinguish T at 3’ end. The reverse primer was common for both the reactions

(Table 2). To increase specificity, an extra mismatch is also deliberately introduced at the third position from the 3' end of each of the two allele specific primers. For UTY19, a common forward primer and two reverse primers capable of distinguishing C & A were designed (Table 2). While designing the primers, oligonucleotide melting temperature, oligonucleotide length, GC content, primer-dimer possibilities, PCR product size, positional constrains within the source sequence were considered. Final reaction mix contained 1X PCR Buffer, 1.5 mM MgCl2, 1.00 U of Taq DNA Polymerase (Sigma Aldrich), 200 µM dNTPs (Sigma Aldrich), 10pM of each primer and 50 ng of genomic DNA in a final volume of 25µl. Amplification was performed on a PTC-200 thermal cycler (MJ research, Inc). Amplification cycles comprised of denaturation at $95^0$ C for 5 min, followed by 30 cycles (in case of ZFY9 and ZFY10) or 33 cycles (in case of DDX3Y1, DDX3Y7 and UTY19) at $95^0$C for 25s, 52–$57^0$C for 15s, $72^0$C for 25s, and a final extension step at $72^0$C for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels, visualized under ultraviolet illumination (UVP Bioimaging Systems, Upland, CA®) and the presence or absence of specific band was indicative of presence or absence of a particular allele (Table 2). A sample was considered negative for a particular allele when the amplicon was absent. Furthermore, same template DNA was used in both reactions of each allele specific genotyping assay which serves as a control for the false positive or negative reaction secondary to extraction failure or the presence of an inhibitor. The sequence of primers, their respective nucleotide numbers, target region and amplicon sizes are listed in Table 2. All the bulls, under study, were screened by using standardized AS-PCR protocols.

**Validation of the assay:** To exclude any false positive results of AS-PCR, arising out of non-specific amplification, three representative amplicons of 259, 286, 249, 282 and 280 bp pertaining to ZFY-9, ZFY-10, DDX3Y-1, DDX3Y-7 and UTY-19 regions were amplified (Gotherstrom *et al.,2005*; Ginja *et al.,*2009) (Table 1). Amplicons were sequenced directly using automated DNA sequencer. Results obtained by Allele Specific PCR and direct sequencing were compared. In all the assays negative (no template) and female DNA (Crossbred and *Bos indicus* cow) were used as control to rule out the possibility of PCR contamination and nonspecific amplification.

## RESULTS AND DISCUSSION

Upon amplification, 259, 286, 249, 282 & 280 bp products corresponding to ZFY-9, ZFY-10, DDX3Y-1, DDX3Y-7 and UTY-19 were obtained (Table 1, Fig. 2). Two female samples of Tharparkar and HF Crossbred as controls were observed to have negative amplification with respect to above five loci (data not shown). Markers on non-recombining region of Y chromosome, paternally inherited in a haploid fashion, have extensively been used for studying the origin of species, range expansion, admixture of populations and migration in animals (Lindgren *et al.,* 2004; Pidancier *et al.,* 2006). As a consequence of single parent inheritance, one of the alternative forms of a putative Y-specific SNP may be available at a time in any individual. Till date, Y-SNPs screening studies are mainly sequencing based, laborious and time consuming. Here we developed an alternative approach i.e., AS-PCR for screening of SNPs at MSY of bull Y-chromosome. In the present investigation, a total of 181 bulls from 10 Indian native breeds and HF crossbred animals were screened for five Y-specific cattle SNPs (*viz.*, ZFY-9:120> C/T; ZFY-10:655> C/T; DDX3Y-1:425>C/T; DDX3Y-7:123>C/T and UTY-19:423>C/A) using standardized AS-PCR protocols (Fig. 3, and 4). The allelic pattern obtained from AS-PCR protocols was further confirmed by direct nucleotide sequencing in representative samples (Fig. 5 and 6). Absolute concordance was observed between results obtained

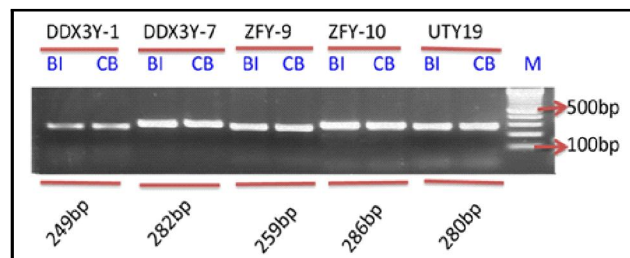


**Fig 2:** Amplification of five target regions of different Y specific genes (DDX3y, ZFY and UTY)

**Table 1:** Amplification of five target regions of bull MSY

| Locus | Intron | Size(bp) | Ta(°C) | Primer sequences (5' -3' ) | SNPs | GenBank# |
|---|---|---|---|---|---|---|
| ZFY-9 | 9 | 259 | 56 | F—TCACATTGCAGCTTTAGGATTG<br>R—CCTTCACTTGGCAGATGGAT | 120>C/T | AY928828 |
| ZFY-10 | 10 | 286 | 52 | F— CCAAAATGGTTGAGCTTTATGA<br>R— GGAGCATAAGTGATCCAATGAA | 655>C/T | AF241271 |
| DDX3Y-1 | 1 | 249 | 58 | F—TGAACCACTAGGGAGGTCATC<br>R-TTCCAATTTAGCTGTGGTTATCTG | 425>C/T | AY928816 |
| DDX3Y-7 | 7 | 282 | 58 | F—TTGAAAGCTGTGAAGGTAGGG<br>R—GATACTGTTTACGGCGTCCA | 123>C/T | AY928819 |
| UTY-19 | 19 | 280 | 52 | F—GATGCCTATATTAGCCATTGACA<br>R—AAATTCTTTATGATGTTCCATCC | 423>C/A | AY936543 |

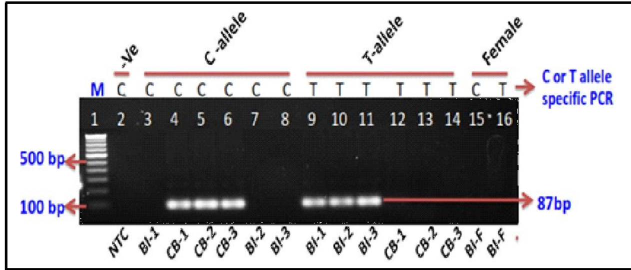


**Fig 3:** Screening of SNP of ZFY-9 (120> C/T) through AS-PCR

Lane 1 : 100 bp DNA Ladder
Lane 2 : Negative control
Lane 3-8 : C allele specific PCR product from CB and BI
Lane 9-14 : T-Allele specific PCR product from BI and CB
Lane 15-16: Female samples from BI cattle; CB: HF crossbred
BI : *Bos indicus*

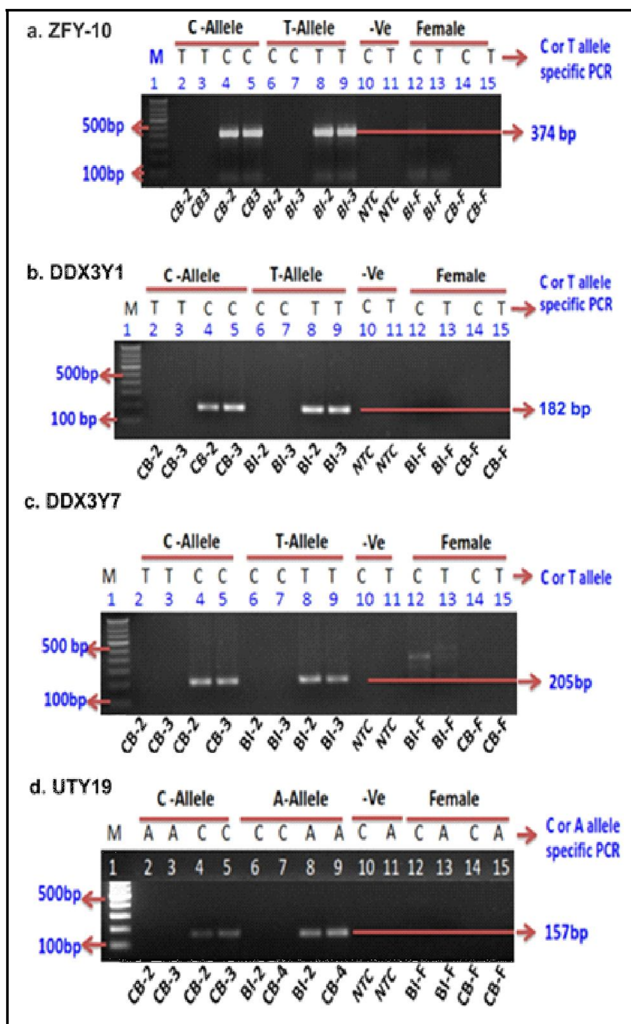


**Fig 4:** Screening of SNP of ZFY-10 (655> C/T), DDX3Y-7 (123> C/T), DDX3Y-7 (123> C/T) through allele specific PCR

Lane 1 : 100 bp DNA Ladder
Lane 2-3 : Negative samples
Lane 4-7 : CB samples
Lane 8-11 : BI samples
Lane 12-15: Female samples from BI and CB cattle
CB : HF crossbred
BI : *Bos indicus*

**Table 2:** Allele specific PCR primers detail for screening five SNPs on bull Y- chromosomes

| SNP | Primer ID | Primer Sequence (5'-3') | Conc (µM) | Ta (ºC) | No. of PCR cycles | Product size (bp) | Reference |
|---|---|---|---|---|---|---|---|
| ZFY9: 120> C/T | AS-ZFY9(T) | TACTAATGAACTGATTTAAGTAAAAAACA | 1.0 | 54 | 30 | 87 | Kumar *et al*., 2015 |
| | AS-ZFY9(C) | TACTAATGAACTGATTTAAGTAAAAAACG | 1.0 | | | | |
| | ZFY9-CR | CATAAAGAAAGTTCCTATTAAAGTTAAA | 1.0 | | | | |
| ZFY10: 655> C/T | AS-ZFY10(C) | TTTAATTATATTTTAATTGGTACAGTCCTC | 1.0 | 52 | 30 | 374 | Kumar *et al*., 2015 |
| | AS-ZFY10(T) | TTTAATTATATTTTAATTGGTACAGTCCTT | 1.0 | | | | |
| | ZFY10-CR | CTTCTTATTGGTAGTGTAATCACAATCA | 1.0 | | | | |
| DDX3Y-1: 425> C/T | AS-DX3Y1(T) | CAAATATTTCTTGACATTAATGTGAT | 1.0 | 52 | 33 | 182 | Kumar *et al*., 2015 |
| | AS-DX3Y1(C) | CAAATATTTCTTGACATTAATGTGAC | 1.0 | | | | |
| | DDX3Y1-CR | TCCAAAAACATAGCTGTATATTAGTAAT | 1.0 | | | | |
| DDX3Y-7: 123> C/T | AS–DX3Y7(T) | TCTTTTTCTATGCATTTCTAAAGGTT | 1.0 | 57 | 33 | 205 | Present study |
| | AS–DDX3Y7(C) | TCTTTTTCTATGCATTTCTAAAGGTC | 1.0 | | | | |
| | AS–DDX3Y-7(CR) | TTGAAAGCTGTGAAGGTAGGG | 1.0 | | | | |
| UTY19: 423> C/A | AS-UTY-19(CF) | TCCAGAGCCTGCTTATGTCC | 1.0 | 57/52* | 33 | 157 | Present study |
| | AS-UTY-19(C) | AAAAATTCTTTATGATGTTCCATTCC | 1.0 | | | | |
| | AS-UTY-19(A) | AAAAATTCTTTATGATGTTCCATTCA | 1.0 | | | | |

*Annealing temperature for C- and A-specific amplicons are 57ºC and 52ºC, respectively

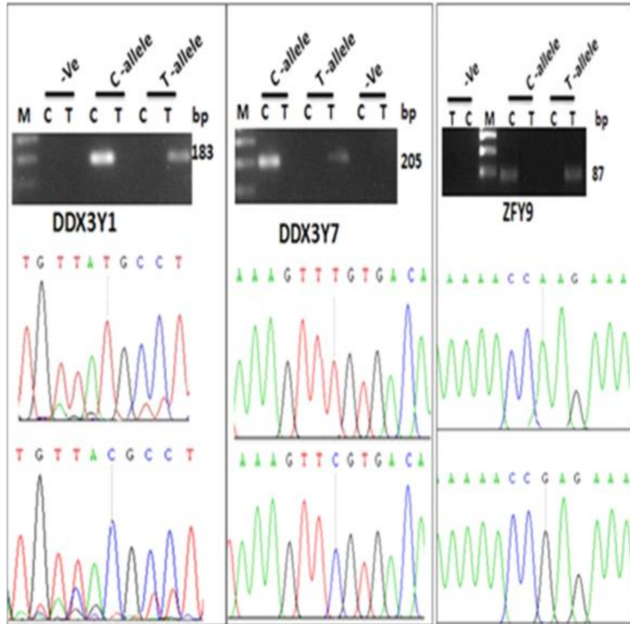


Fig 5: Validation of allele types through direct sequencing of representative samples pertaining to ZFY-9, DDX3Y-1 and DDX3Y-7

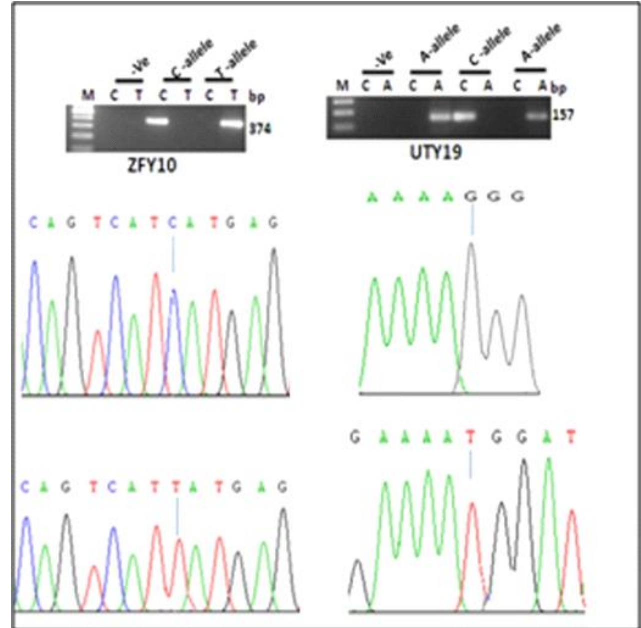


Fig 6: Validation of allele types through direct sequencing of representative samples pertaining to ZFY-10 and UTY-19

through direct sequencing and allele specific-PCR. Moreover, in every assay one or two negative (no template) and female DNA used as controls revealed no template contamination and nonspecific amplification. While using AS-PCR genotyping assays one critical point to remember is that mismatches may extend at a lower rate, resulting in an amplification delay. Hence, careful optimization of number of PCR cycles in each assay has been standardized (Table 2) as excess number of cycles could result in poor genotyping discrimination. High-resolution melting (HRM) Real Time PCR assay based screening of Y-chromosome variations is another simple and reliable alternative (Ganguly *et al*., 2016). Y-SNPs haplogroup analysis based on above five SNPs has provided powerful information about the domestication and genetic structure of cattle worldwide. Previous studies (Gotherstrom *et al.,* 2005; Ginja *et al.,* 2009) have identified three different haplogroup (Y1 and Y2 in *B. taurus* and Y3 in *B. indicus*) in contemporary cattle, Where Y2 haplogroup represents the taurine cattle domesticated in the Fertile Crescent, the Y1-haplogroup generally represents European aurochs. Y1 is predominant in northern Europe and in a number of Iberian breeds. In contrast, Y2 is dominant in most central, Mediterranean and Iberian breeds. In addition, Y2 is also observed in several British and Nordic breeds (Gotherstrom *et al.,* 2005 Kantanen *et al.,* 2009; Ginja *et al.,* 2010; Edwards *et al.*, 2011). Although in cattle breeds of Central Asian countries (Kazakhstan, Turkmenistan and Kyrghyz) as well as in Africa, both the Y1 and Y2 lineages have been observed (Perez-Pardal *et al.,* 2010; Edwards *et al.,* 2011); however, only Y2 haplogroup has been reported in the breeds of East Asian Countries like Korea and Japan (Perez-Pardal *et al.,* 2010). In the Chinese, *Bos Taurus* (Y2) *and Bos indicus* (Y3) origin specific lineages have been reported (Chang *et al.,* 2011; Zhang *et al.,* 2013). Subsequent investigations using Y-SNPs and Y-STRs further confirmed that Y2 dominates the north (91.4%) and Y3 in the south of China (90.8%) (Li *et al.,* 2013). All the three haplogroups described in cattle (Y1, Y2 and Y3) have also been detected in Southwest Asia, where Y3 is found to be limited in *Bos indicus* of Indian subcontinent and China (Edwards *et al.,* 2011; Chang *et al.,* 2011; Zhang *et al.,* 2013; Li *et al.,* 2013). In the present investigation three haplogroup (Y1, Y2 and Y3) have been detected (Table 3). While Y1 and Y2 were found in HF crossbred bulls, Y3 was restricted to Indian native cattle breed. In HF crossbred bulls, frequencies of Y1 and Y2 are observed to be 0.98 and 0.02; respectively (Table 3). One possible reason for the higher frequency of Y1 haplogroups observed in the present study may probably because of predominant nature of Y1 lineage in HF bulls (Gotherstrom *et al.,* 2005; Edwards *et al.,* 2011) used in

**Table 3:** Y-chromosome haplogroups of HF Crossbred and *Bos indicus* bulls

| Haplogroups | Origin | ZFY-9 | ZFY10 | DDX3Y1 | DDX3Y7 | UTY19 | Frequency |
|---|---|---|---|---|---|---|---|
| Y1 | Crossbred | C | C | C | C | C | 0.98 |
| Y2 | Crossbred | C | C | C | C | A | 0.02 |
| Y3 | *B.indicus* | T | T | T | T | A | 1.00 |

crossbreding. All the Indian native breeds showed only Y3 haplotype, revealing a pure indicine lineage. The results are in agreement with previous reports, with limited bull samples/ breeds from India or Indian subcontinent (Gotherstrom *et al.,* 2005; Edwards *et al.,* 2011) confirming the zebu morphology.

## CONCLUSION

In summary, AS-PCR screening and analysis of 181 bulls in the present investigation revealed three haplogroups (Y1, Y2 and Y3). Indian native cattle breeds had pure indicine paternal origin (Y3). Whereas, HF crossbreds showed both the *Bos taurus* (Y1 and Y2) paternal lineages with predominance of Y1 (0.98) haplogroup. These AS-PCR protocols may be useful in the screening of Y-SNPs/ haplogroups in various native cattle breeds, exotic and crossbred cattle populations.

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