SEQUENCE CHARACTERIZATION AND POLYMORPHISM DETECTION IN THE BUBALINE CD14 GENE

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ABSTRACT

The present study was undertaken with the objectives of sequence characterization and identification of polymorphisms in the bubaline CD14 gene. Single strand conformation polymorphism analysis revealed a total of eight different variants *viz*. CD14-A, CD14-B, CD14-C, CD14-D, CD14-E, CD14-F, CD14-G and CD14-H in exon 2 of the CD14 gene for four breeds of buffalo. Variant CD14-A was observed to be the wild type as it exhibits highest frequencies, whereas CD14-D was observed to be genetically distant from others. Polymorphism or variability may be regarded to be the highest in the Mehsana breed of buffalo, which exhibited six genotypes out of eight. Thus 42 SNPs were identified for the CD14 gene of buffalo. The CD14 gene ranging from the 592nd to the 856th nucleotide of the second exon, which corresponds to the 198th to the 285th codon of the coding sequence was found to be highly polymorphic and may be regarded as the '*mutational hot spot'*, or the hyper variable site, leading to ligand diversity. Comparison of nucleotide sequences of different regions of the buffalo CD14 gene with that of taurine cattle revealed a total of 22 point mutations, of which eleven were non-synonymous codon with eleven amino acid substitutions. The 258 bp fragment of the CD14 gene ranging from the 3rd nucleotide to the 261st nucleotide of the 2nd exon of the CD14 gene revealed identical nucleotide sequences, indicating monomorphism.

Keywords: CD14 gene, buffalo, exon, polymorphism, SSCP

INTRODUCTION

Mastitis is one of the most common diseases affecting dairy cattle and buffaloes causing huge economic losses to dairy farmers. In India, about 3.84% and 24.43% of buffaloes, respectively, are affected with clinical and subclinical mastitis every year (Singh and Singh, 1994). The annual loss due to clinical and subclinical mastitis in buffaloes was estimated to be Rs. 6,962,900,000 and Rs. 17,233,200,000, respectively (Dua, 2001). Incidences of diseases of milch animals is critical to dairy industry, wreaking economic havoc in terms of veterinary and treatment costs, reduced milk production in the subsequent lactation, milk condemnation due to antibiotic residues, early culling, extra labour involvement and deterioration in milk quality. Antibiotic therapy and vaccination have their own limitations. Thus, it seems

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understanding and subsequent manipulation of the host immune response is the most precise and effective tool to lower the disease incidences and to nullify the limitations associated with antibiotic treatment or vaccination. Selective breeding of dairy cattle and buffaloes for reduced susceptibility to/increased resistance against mastitis is difficult as it is a polygenic trait with very low heritability. However, indirect selection based on somatic cell count and candidate gene markers can help to increase the efficiency of such breeding programs. A fair amount of genetic research related to udder health has already been performed due to its importance from the economic as well as from the quality control points of view (Ogorevc *et al*., 2009)

CD14 is an important molecule for innate immunity. The CD molecule ranges from 1 to 166 with differential structure and functions (Goldsby *et al*., 2000), of these CD14 is the most important molecule known so far, playing a vital role against several endotoxigenic bacteria. Its pattern recognition receptor binds mainly with LPS (lipopolysaccharide), lipotechoic acid, arachidonic acid and thus releases various cytokines which acts for the body's defence. The body's immunity thus can act against a wide range of pathogens including gram-negative bacteria and gram positive as *Mycobacterium* sp., *Pseudomonas* sp. and *Staphylococcus aureus* etc. CD14 functions both as a cell membrane receptor and a soluble receptor for bacterial LPS. It has been considered as an important molecule for its role in various diseases, like mastitis (Lee *et al*., 2003), treponemiasis (Schroder *et al*., 2000) and glomerulonephritis (Yoon *et al*., 2003). Soluble CD14 enriched bovine colostrums and milk induces B cell growth and differentiation (Fillip *et al.*, 2001). The CD14 gene has been cloned and sequenced in other ruminants (Pal and Chatterjee, 2009a; Pal *et al*., 2008)

Detection of SNPs is useful for analysis of the evolutionary history of species development, assessment of biodiversity, associative studies between polymorphisms and disease resistance. The variability at the nucleotide level of the CD14 gene leads to the variability in the CD14 encoded molecule, which in turn gives rise to the phenotypic variability in host immune response. Thus, the variants of the CD14 gene and their association with the incidences of disease occurrence may be used as a marker for disease resistance. Association of polymorphic candidate genes with economic traits will help the breeders to search some genetic markers for economic traits. This may be used as an aid to the selection of bulls at an early age and can save huge economic loss for rearing the bulls till maturity. In spite of its tremendous potential to be exploited, very little work has so far been done whereas such type of marker trait association studies have also been documented by a number of workers in various ETLs (Pal *et al.,* 2004; Pal *et al.,* 2005) No reports are available so far for genetic polymorphism of the CD14 gene in any animal at the coding region; however CD14 gene polymorphism study at the promoter region is available in human. In the last decade, many fine mapping experiments have resulted in identification of several QTLs in cattle affecting milk production and udder health emphasizing their potential value in marker assisted selection programs. However, beyond fine mapping, the ultimate target of QTL analysis is the identification of casual gene itself which would facilitate identification of resistance genes and alleles (Reinard and Riollet, 2005). Scanty reports are available so far for genetic polymorphism of the CD14 gene in an animal at the coding region; however CD14 gene

polymorphism study at the promoter region is available in human (Hubacek *et al*., 1999; Hartel *et al*., 2004; Guerra *et al*., 2004).

Buffaloes are economically important animals with higher milk producing ability (54%) with high SNF and fat percentage, better capacity to utilize coarse fodders and their innate resistance to a wide range of diseases (Annon, 2001). Most Indian buffaloes are of the riverine type; there are a few swamp buffaloes in the northeastern region. So far no reports are available regarding molecular characterization of the CD14 gene in buffaloes and very scanty reports are available regarding SNP detection in any farm animal.

Keeping the above facts in view, the present investigation was planned to identify the polymorphism of the CD14 gene of buffalo, analyze the sequences of the identified variants and compare them with those of other species.

MATERIALS AND METHODS

Animals

Blood samples were collected from a total of 246 unrelated animals belonging to four different breeds of Indian riverine buffaloes (*Bubalus bubalis)* with Murrah (103), Mehsana (69), Surti (36) and Bhadawari (38). Buffaloes were maintained at different government farms in India as well as farmers'herds, gaushals and NGOs. Samples from Murrah buffalo were collected from farmers' herds, gaushals and NGOs at Dubrajpur Block, Birbhum district, West Bengal and the Indian Veterinary Research Institute, Izatnagar (U.P.). Samples from Mehsana and Surti buffaloes were collected from Gujrat Agricultural University, S.K. Nagar (Gujrat), Gujrat Agricultural University, Navasari, Anand (Gujrat), and the Government Livestock Farm, Etawah (U.P.) respectively .

Blood was collected from the jugular vein into EDTA containing vacutainer tubes and DNA extraction was performed from whole blood following a standard phenol-chloroform extraction method (Sambrook and Russell, 2001).

Amplification of the Bubaline CD14 gene

The 2nd exon of CD14 gene was studied in two fragments. A 258 bp fragment (the 3rd nucleotide to the 261st nucleotide of CD14 cDNA) of the coding sequence of Bubaline CD14 (the 587th to the 854th nucleotide of second exon) was amplified using the forward primer as ATGGTGTGCGTGCCCTACCTG and the reverse primer as TATGCTGACACAATCAAGGCT and the fragment obtained was of 258 bp. The reaction mixture used was PCR buffer 1.2X, $MgCl_2$ 1.5 mM, dNTP 0.5 mM, forward primer and reverse primer 60 ng each, Taq DNA polymerase 1 unit. The reaction condition used was initial denaturation at 94^{o}C for 5 minutes, denaturation at 95^{o}C for 45 seconds, followed by annealing for 61^{o}C for 30 seconds, extension for 72^{o}C for 45 seconds, repeated for 34 cycles followed by final extension for 72^{o}C for 7 minutes.

The second fragment amplified was a 265 bp fragment of the coding sequence of Bubaline CD14 (the 587th to the 854th nucleotide of the second exon). PCR was carried out in a final volume of 25 µl of reaction mixture containing 80-100 ng DNA, PCR buffer 1.2X, $MgCl_2$ 1.5 mM, dNTP 0.5 mM, forward primer and reverse primer 60 ng each, Taq DNA polymerase 1 unit. The DNA was subjected to amplification with polymerase chain reaction in a thermocycler (PTC-200, MJ Research, USA) using the forward primer as AGCGAACGACAAATTGAGAGACCTTAGTG and the reverse primer as AAGGTCTCTCAATT

TGTCGTTCGCTGGGC and the fragment obtained was of 265 bp. The reaction condition used was initial denaturation at 94°C for 5 minutes, denaturation at 95°C for 45 seconds, followed by annealing for 61°C for 30 seconds, extension for 72°C for 45 seconds, repeated for 34 cycles followed by final extension for 72°C for 7 minutes.

Genotyping by PCR-Single stranded conformation polymorphism

SNPs were detected by PCR-SSCP and identified by subsequent sequencing. PCR-SSCP was performed for all the samples. In 0.5 ml PCR tubes, 3 µl of amplified PCR-product was mixed with 9 µl of formamide dye.. This 12 µl of PCR-SSCP solution was denatured at 95°C for 5 minutes followed by immediate chilling in ice for 15 minutes. The products were run in 12% polyacrylamide gel at 4°C for 11 h at 200 V in case of the 268 bp fragment and 6 h for the 261 bp fragment. SSCP fragments were visualized by silver staining and documented in the Gel Documentation System. Silver staining was carried out according to the procedure described by Basam *et al.* (1991) with some modifications.

The variants were detected directly by observing the SSCP pattern of each sample in the polyacrylamide gel. PCR products from different variants were selected and sequenced using respective forward and reverse primer to detect variations if any, at the nucleotide level. Then sequences were aligned with those of the reported CD14 sequences of different species using MegAlign Programme of Lasergene Software (DNASTAR). Moreover, sequence variations were also compared with the cDNA (Gene bank acc no. DQ457089) and genomic DNA of buffalo (Gene bank acc no. DQ444324), we have reported earlier. A phylogenetic tree was constructed for detecting the genetic distance between different variants and their evolutionary significance. (MegAlign Programme of Lasergene Software, By ClustalW Method, DNASTAR).

The genotypes were detected directly by observing the SSCP pattern of each sample in the polyacrylamide gel. The gene and genotype frequencies were estimated by a direct counting method (Falconer and Mackay, 1998). PCR products from different genotypes were selected and sequenced using respective forward and reverse primers to detect variations if any, at the nucleotide level. Then sequences were aligned with those of the reported CD14 sequences of different species using MegAlign Programme of Lasergene Software DNASTAR, Inc, Madison WI, USA). Multiple sequence alignments were performed with the Megalign. program of LASERGENE software. The coding DNA sequences of different exonic regions were conceptually translated to amino acid sequences using the same software.

RESULTS AND DISCUSSION

PCR-SSCP analysis of 258 bp fragment

The 258 bp fragment from the 3rd nucleotide to the 261st nucleotide of the 2nd exon of CD14 gene (Figure 1) revealed identical nucleotide sequences (Gene bank acc no. EU370404), indicating monomorphism of the gene (Figure 2). This indicates the conserved nature of the CD14 gene of buffalo pertaining to this region. It reveals the conserved nature of this gene for the first 258 nucleotides, encoding for 86 amino acids. This region in buffalo may probably be under strong purifying selection which may explain the lack of SNPs in this region. This may be the reason that 1

to 60 nucleotide codes for signal peptide, when no variation is expected. The present finding is similar to CB cattle (Pal and Chatterjee, 2009b), where monomorphism was reported for this nucleotide region except for one nucleotide change.

PCR-SSCP analysis of 265 bp fragment

The amplified product of the 265 bp fragment of the CD14 gene was observed by agarose gel electrophoresis (Figure 3). Eight variants designated as CD14-A, CD14-B, CD14-C, CD14-D, CD14-E, CD14-F, CD14-G and CD14-H were detected in 265 bp fragment of CD14 gene of buffalo. The frequencies of the different variants of bubaline CD14 gene are depicted in Table 1. In the Bhadawari breed of buffalo, only three variants were identified as CD14-A, CD14-B and CD14-F (Figure 4) with the respective frequencies being 0.818, 0.045 and 0.136 (Figure 5). In the Mehsana breed, all variants, except CD14-D and CD14-H were identified (Figure 6). The highest frequency was observed for the CD14-A genotype (0.465), whereas CD14-B (0.056) and CD14-G (0.056) were found to be the least frequent genotypes (Figure 7). The Murrah breed revealed only four patterns (Figure 8) with CD14-A being the most frequent (0.596), whereas CD14-B (0.105) and CD14-D (0.105) were the least frequent genotypes (Figure 9). The Surti breed of buffalo showed a different picture where the highest frequency was observed for the CD14-D genotype, and the CD14-A (0.071) and CD14-H (0.071) genotypes were the least frequent (Figure 10, Figure 11). Since this is the first report of study for polymorphism of the CD14 gene in farm animals and first time in the coding region in any animal so far, comparison was not possible. However, polymorphism has been identified in the promoter region of the CD14 gene in human being at C (-260)→T (Hubacek *et al*., 1999), (-159) position (Hartel *et al.,* 2004, Guerra *et al.*, 2004), (-1619), (-550) from the transcription start site of the CD14 gene (Guerra *et al.*, 2004).

CD14-E may be considered as the breed specific marker for the Mehsana breed. whereas CD14-G and CD14-H may be considered as breed specific markers for the Mehsana and the Surti, repectively. In the present study, the Mehsana breed exhibited six genotypes out of eight. Thus polymorphism or variability may be regarded to be the highest in this breed. Simultaneously heterozygosity may also be expected to be high in this breed.

The frequencies of different variants estimated in the population of four different breeds are listed in Table 1 and Figure 12. The most frequent variant (genotype) was identified as CD14-A, with the overall genotypic frequency being 0.47. The least frequent were CD14-G and CD14-H, with very low frequencies (0.018) for both the genotypes. The frequencies for other genotypes were intermediate. Thus CD14-A may be the original wild type allele for the gene. The other variants may therefore be the result of recent mutational events. Among the breeds, Bhadawari had the highest frequency (0.818) for CD14-A and Surti had the lowest frequency for this pattern (0.071) which showed the highest homozygosity in the Bhadawari population and least in the Surti population.

CD14-D was observed to be genetically distant from other variants as evidenced from Figure 13. Since the Surti breed of buffalo was observed to have highest frequency for CD14-D (Figure 11), it is expected that the Surti is genetically distant from the other breeds.

Sequence analysis of PCR-SSCP 265 bp fragments

Six different SSCP variants CD14-A (Gene bank acc no. EU370398), CD14-B (Gene bank acc no. EU370399), CD14-C (Gene bank acc no. EU370400), CD14-D (Gene bank acc no. EU370401), CD14-E (Gene bank acc no. EU370402), CD14-F (Gene bank acc no. EU370403) were sequenced for determining the variation at the nucleotide level. The combined effects of the SNPs were estimated through PCR-SSCP. Moreover, sequence variations were also compared with the cDNA (Gene bank acc no. DQ457089) and genomic DNA of buffalo (Gene bank acc no. DQ444324), we have reported earlier. Since this is the first report of CD14 gene polymorphism in coding region, comparison was not possible. However, polymorphism studies have been conducted at promoter region in human (Hubacek *et al.,* 1999; Guerra *et al*., 2004; Hartel *et al*., 2004; Geaghan *et al*., 2010). The SNPs have been depicted in Figure 14. A very high degree of nucleotide sequence variability was observed for CD14-B (81.3), CD14-C (97.0), CD14-D (86.0), CD14-E (85.8) and CD14- F (85.8) as compared to CD14-A (Table 2). A high degree of nucleotide variation was observed between the alleles/patterns identified within the coding sequence of CD14 gene for the 265 bp fragment ranging from the 587th to the 854th nucleotide the of second exon of buffalo, which corresponds to the 197th to the 285th codon of the coding sequence.

Phylogenetic analysis of CD14 gene of riverine buffalo

The percent homology of different regions of the bubaline CD14 gene with that of various species is presented in Table 3. The sequence homology of the 258 bp region was 97.7% with *Bos taurus*, 95% with *Bos indicus*, 96% with *Bos grunniens*, 91.5 with *Capra hircus* and 94.6 with *Ovis aries*. The percent sequence similarity of the bubaline 265 bp region was greater than 90% with ruminants (*Bos taurus*, *Capra hircus* and *Ovis aries*) while it was less than 90% with monogastric species (*Sus scrofa*, *Canis familiaris* and *Equus caballus*). Similarly, the percent homology of bubaline CD14 was more than 90% with ruminants while it was 84% with *Equus caballus*. Comparative analysis of the buffalo CD14 peptide sequence with that of cattle has been depicted in Table 4. This represents the species specificity. Phylogenetic closeness of buffalo with cattle as observed in the present study has also been reported while studying other genes like growth hormone gene (Pal and Chatterjee, 2010). Chicken has been found to be genetically most distant to buffalo.

SNP detection of bubaline CD14 gene

Very high degrees of nucleotide variation with 42 SNPs were identified with 39 non-synonymous changes, leading to 23 amino acid changes. Twenty-nine transversional mutations and 18 transitional mutations were detected (Table 5). At some sites, both transitional and transversional mutations were detected: at 702, 703, 764, 767, 770 nucleotide positions of CD14 gene of buffalo. Non-synonymous substitutions exceeding synonymous substitutions indicates the evolution of this protein through positive selection among domestic animals. SNPs for the CD14 gene of buffalo was reported here for the first time and moreover, this is the first report of SNPs in any farm animal. Combined genotypes of SNPs were analyzed using a PCR-SSCP method. Thus, the 268 bp fragment of the coding sequence of Bubaline CD14 was found to be highly polymorphic and may be regarded as the 'mutational hot spot' or the hyper variable site,

leading to ligand diversity. Similar higher degrees of mutations have been observed in some other genes as 25 SNPs in the leptin gene in Korean cattle (Chung *et al*., 2008), 96 SNPs from TLRs and their associated intracellular signaling molecules (Dhiman *et al*., 2008) in human.

Most of the SNPs identified were within LRR at amino acid positions 209, 210, 215, 217, 246, 249, 252, 255, 256, 257, 266, 267, 271, 273, 274, 275, 277, 280. It has been reported that LRR in the extracellular domain is responsible for the recognition of pathogens and participates in receptor and ligand interactions (Gordon, 2002). Grooves within the CD14 molecule were responsible for receptor recognition and binding (Kim *et al*., 2005). Since CD14 molecules can bind to a wide range of substances including lipopolysaccharides from gram-negative bacteria, lipo-arabinomannans of mycobacteria, manuronic acid polymers of *Pseudomonas* sp. and to peptidoglycans of *Staphylococcus aureus*, it is expected that there should be sufficient variabilty in the pathogen recognition and receptor binding site, which is primarily comprised of LRR region of the CD14 molecule (Kim *et al*., 2005). Thus, the presence of the LRR coding region may be the region for maximum variability to enable the CD14 molecule to bind with a wide range of substances. From the sequence alignment studies, it was observed that the particular leucine moeity was almost unaltered within the leucine rich repeats and the variations were observed for other amino acids. Thus the basic function of leucine rich repeats remains unaltered.

However, in the absence of any available report regarding the polymorphism study of this particular region in livestock species, comparison is not possible. Moreover, similar nucleotide variations were also observed for this particular region of the CD14 gene between different species, which may give some insight.

Further research need be directed to finding out the association of allelic variants with traits related to disease incidences, to establish markers. There is a need to ascertain the differential biological potency of different allelic variations of CD14. On the basis of such functional study, the most potent variant can be expressed *in vitro* for further therapeutic or immuno competence study. Gene inserts containing the resistant variety of CD14 gene of buffalo may be approached for somatic gene therapy, particularly against mastitis. Transgenic animal production with the buffalo CD14 gene insert may provide scope for future research to develop disease resistant stock.

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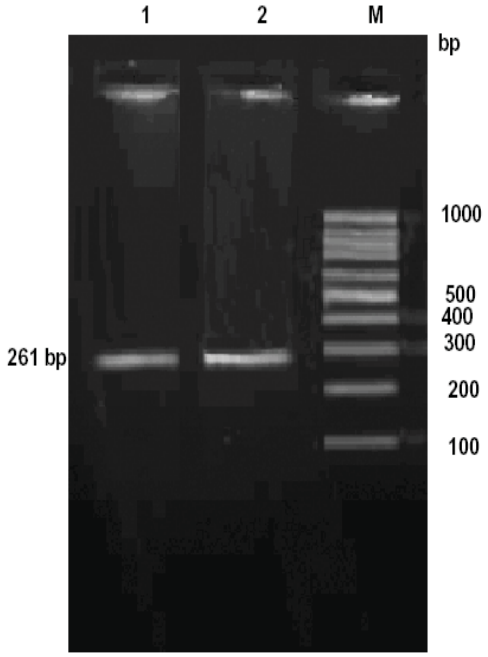

Figure 1. Amplification of the 258 bp fragment of the CD14 gene of buffalo.
Lane 1: Amplified product of 258 bp fragment.
Lane M: 100 bp DNA ladder.

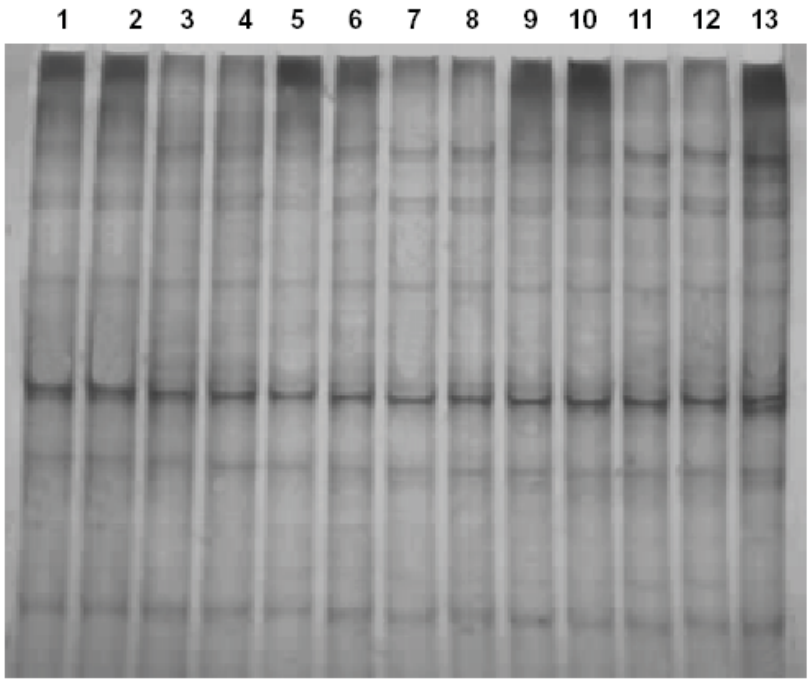

Figure 2. PCR-SSCP pattern of the 258 bp fragment of the CD14 gene of buffalo.

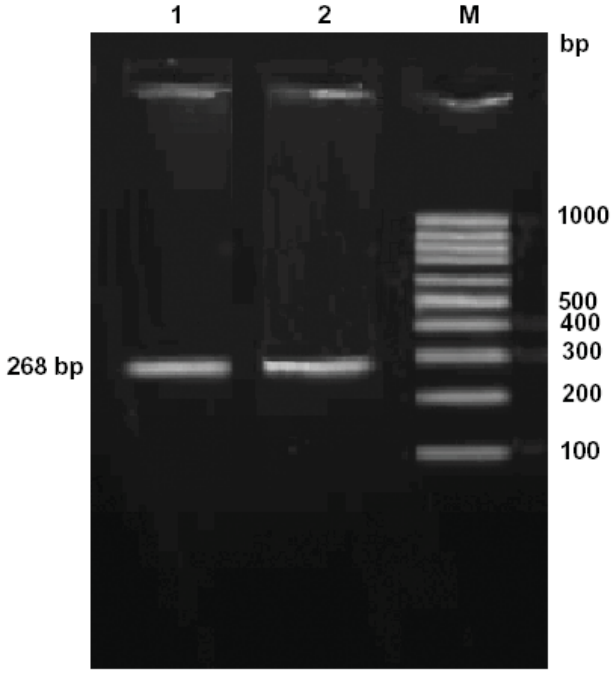

Figure 3. Amplification of the 265 bp fragment of the CD14 gene of buffalo.
Lane 1: Amplified product of 265 bp fragment.
Lane M: 100 bp DNA ladder.

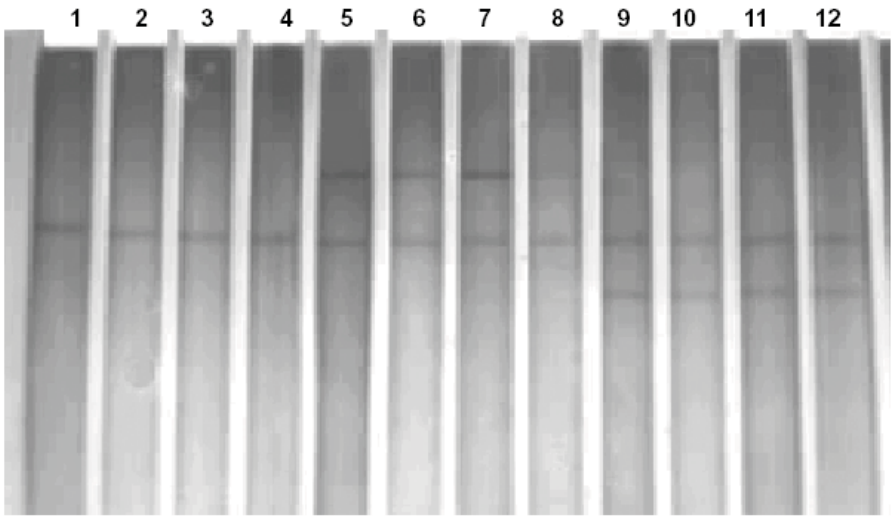

Figure 4. PCR-SSCP pattern of the 265 bp fragment in the Bhadawari buffalo CD14 gene in 12% PAGE.
Lane 1-4 and 8: CD14-A. Lane 9-12: CD14-F pattern.
Lane 5-7: CD14- B.

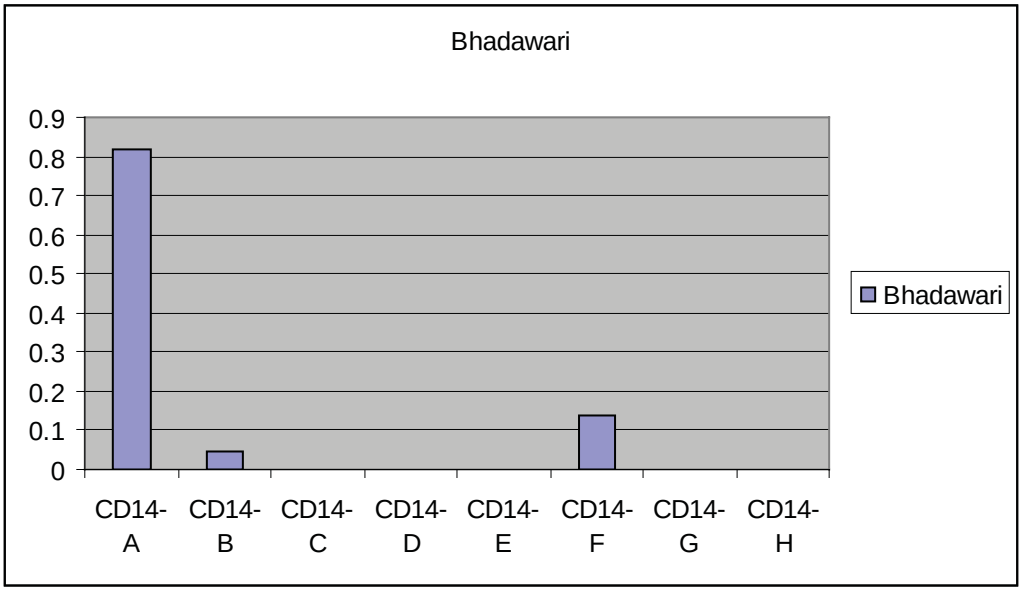

Figure 5. Frequency distribution for different SSCP variants in the Bhadawari breed of buffalo.

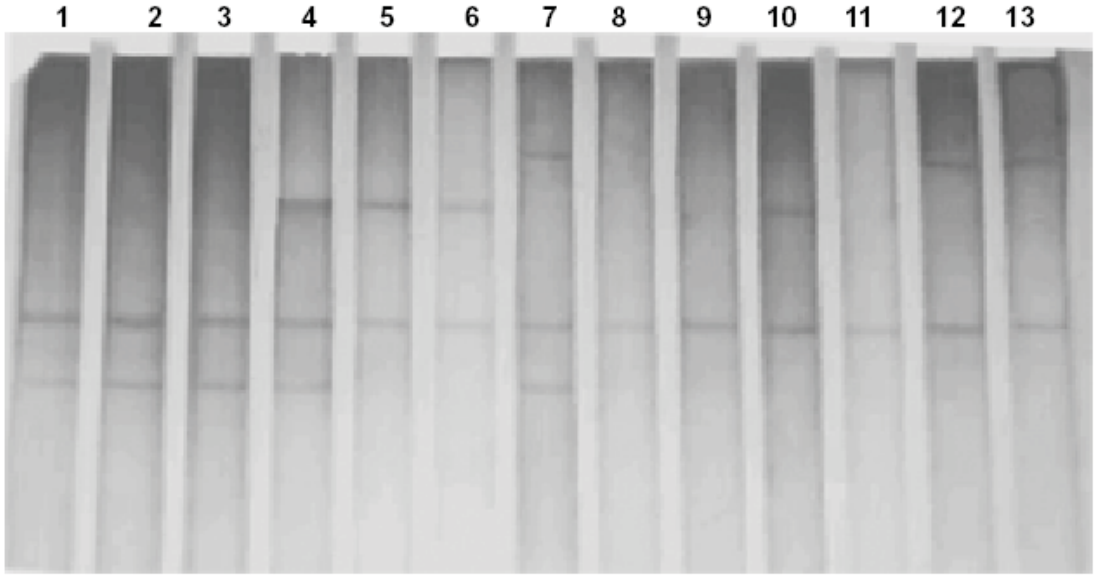

Figure 6. PCR-SSCP pattern of the 268 bp fragment in the Mehsana buffalo.

Lane 1-3: CD14- F. Lane 4: CD14- G.
Lane 5, 6, 10: CD14- B. Lane 8, 9,11 : CD14- A.
Lane 12-13: CD14- C. Lane 7: CD14- E.

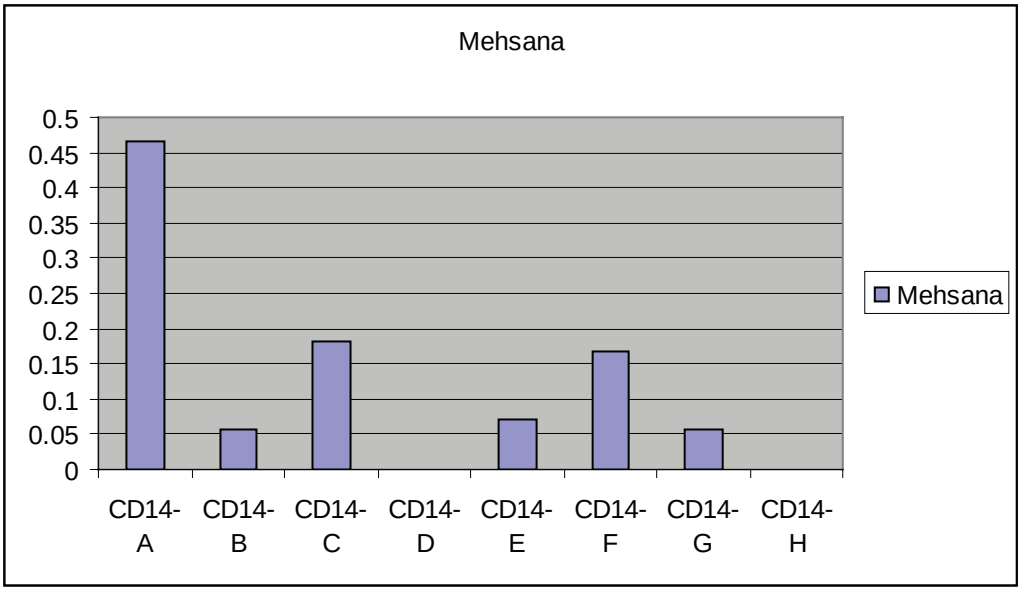

Figure 7. Frequency distribution for different SSCP variants in the Mehsana breed of buffalo.

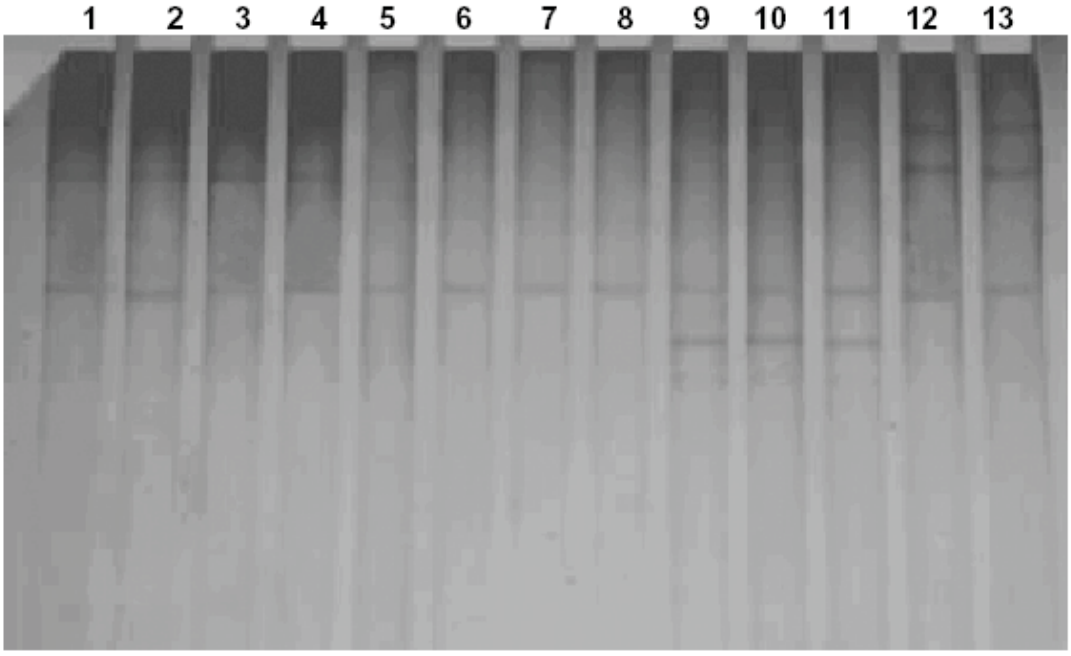

Figure 8. PCR-SSCP pattern of the 265bp fragment in the Murrah buffalo.
Lane 5-8: CD14-A. Lane 1-4: CD14- B.
Lane 12-13: CD14- D. Lane 9-11: CD14- F.

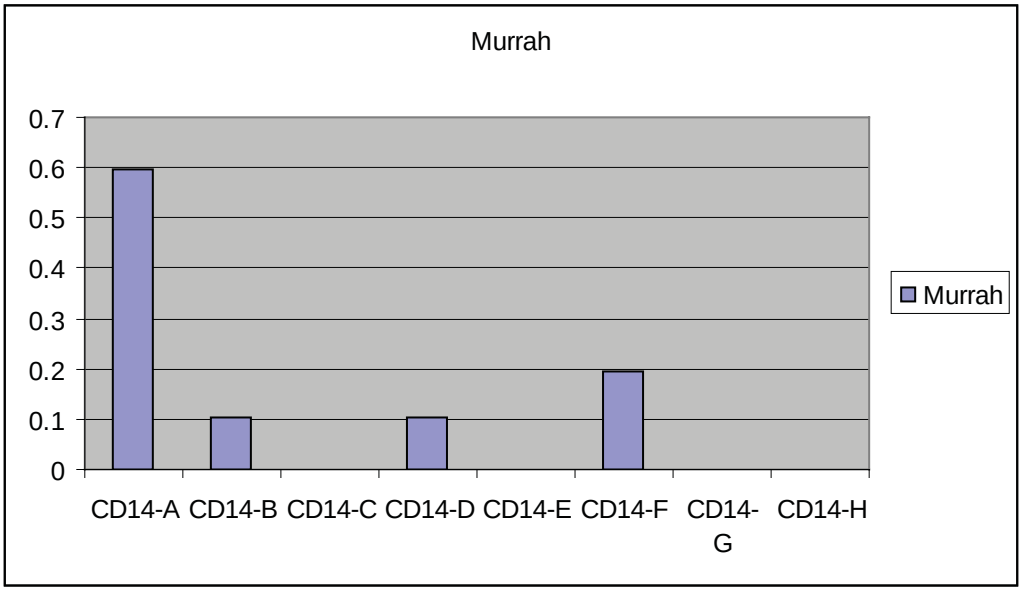

Figure 9. Frequency distribution for different SSCP variants in the Murrah breed of buffalo.

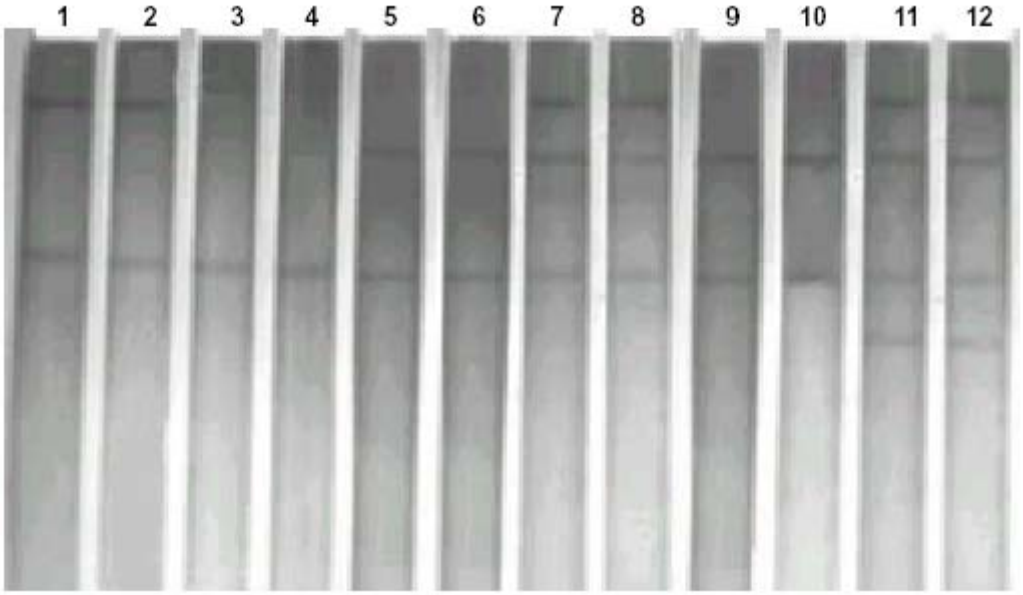

Figure 10. PCR-SSCP pattern of the 268 bp fragment in the Surti buffalo.

Lane 1-2: CD14- C.
Lane 3-4: CD14- A.
Lane 5-6, 9-10: CD14- B.
Lane 7-8: CD14- D.
Lane 11-12: CD14- H.

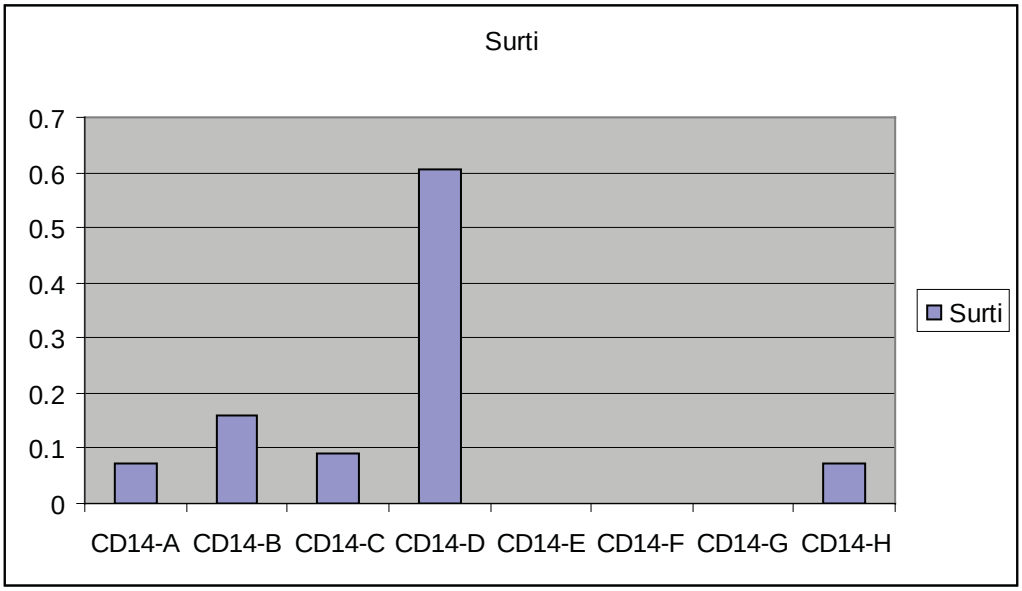

Figure 11. Frequency distribution for different SSCP variants in the Surti breed of buffalo.

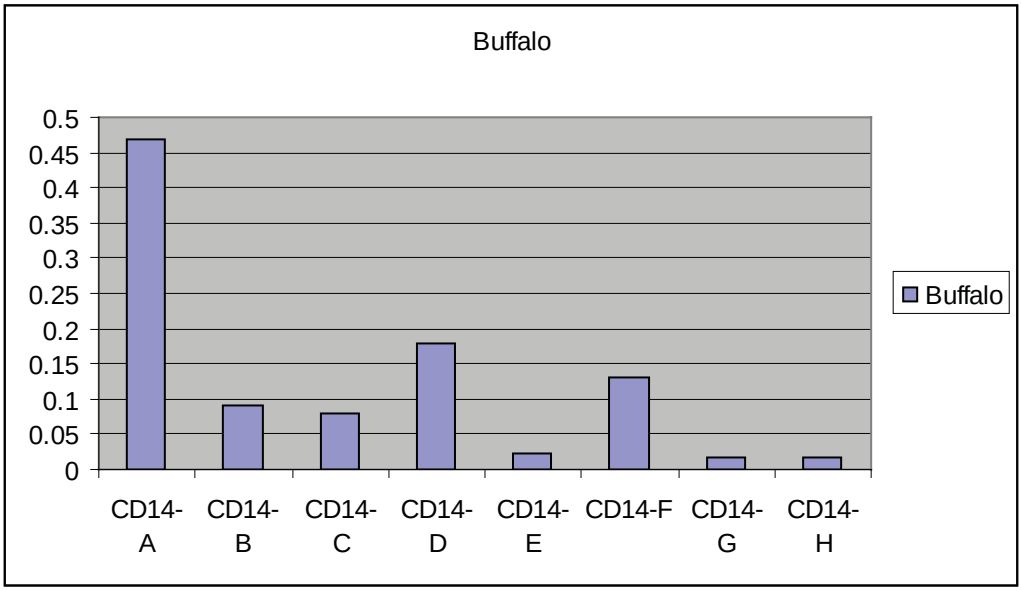

Figure 12. Frequency distribution for different SSCP variants of buffalo.

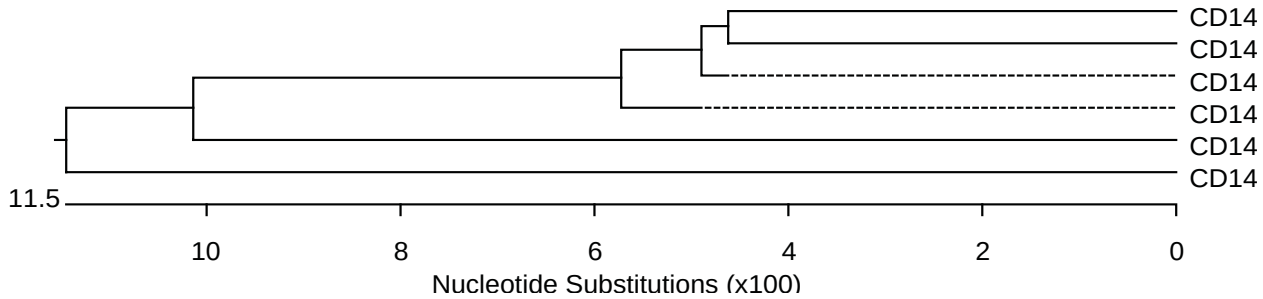

Figure 13. Phylogenetic tree constructed for variants of CD14 gene in riverine buffalo.

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  1 C T A G A C C T G T C T G A C A A T C C C A G T C T C/A G G/C C
 31 G/C A C/G A/G C C G/T G G/T C T G A T G G C A G C T C T C T G T C C/T G/A
 61  A A C A A/G G T T C C C G G C C C T C C A A T A/G T C T A G C G/A
 91 C/G T A/C C G C A/C A C/T G/C C C G G G/T A/C T G/A G /A A G/A/C A/G C/A G     CTG AG C
121 G G A G T G T G C G C G G C G C T G G C G/T G C A G/A C/G G A G/A G
151 G/C T G C A/C G/T C/G C C C A A/T A/C G C C T/G G G A/C C C T /A/C C A G/A C C A/G/C C
181 A/T A/G C/T T/C C/T G C T G C G/A C G T C A/T/C C/G C G/A C C C C G/C G G/C C G C/A T/G
211 A C C C G A/T T/A G/A T/G G T C/A T G/A G/C C/G C C/A A G T G C/T A/G C T A A G/T G
241 T C T C T C A/G A T T T G T/G C G T/C T C G C T G G G C
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Figure 14 . SNPs for different sscp fragments (265 bp) of CD14 gene in buffalo.

Table 1. Frequencies of the identified variants of the 265 bp fragment of the CD14 gene in buffalo.

Breed	CD14-A	CD14-B	CD14-C	CD14-D	CD14-E	CD14-F	CD14-G	CD14-H
Bhadawari	0.818	0.045	-	-	-	0.136	-	-
Mehsana	0.465	0.056	0.183	-	0.070	0.169	0.056	-
Murrah	0.596	0.105	-	0.105	-	0.193	-	-
Surti	0.071	0.16	0.089	0.607	-	-	-	0.071
Overall	0.47	0.09	0.08	0.18	0.022	0.13	0.018	0.018

Table 2. Genetic identity of different variants of CD14-A with respect to others.

SSCP variants	*Gene bank* Accession no.	Percent identity
CD14-B	EU370399	81.3
CD14-C	EU370400	97.0
CD14-D	EU370401	86.0
CD14-E	EU370402	85.8
CD14-F	EU370403	85.8

Table 3. Percent sequence homology of amplified region of bubaline CD14 gene with various species.

	258 bp 3rd nucleotide to 261st nucleotide		265 bp 587th to 854th nucleotide of second exon	
Species	**Accesion**	**Percent homology**	**Accesion**	**Percent homology**
Cattle	NM_174008	97.7	NM_174008	98.1
Goat	DQ457090	91.5	DQ457090	90.6
Sheep	AY289201	94.6	AY289201	96.6
Human	NM_000591	76.7	NM_000591	80.8
Pig	EF051626	80.6	EF051626.	81.5
Rabbit	M85233.1	77.1	M85233.1	73.2
Mouse	NM_009841	62	NM_009841	75.5
Rat	NP 068512	66.7	NP 068512	73.2
Horse	AF200416	80.2	AF200416	84.5
Dog	XP_848746	79.1	XP_848746	77.7
Chicken	AM933591	15.9	AM933591	19.2
Monkey	XP_517975	76.0	XP_517975	80.4

Table 4. Comparative analysis of the buffalo CD14 peptide sequence with that of cattle.

Sl. No.	Amino acid sequence position	Buffalo	Cattle	Nucleotide substitution
1	14*	Proline	Serine	C/T
2	62*	Glycine	Alanine	G/C
3	209**	Serine	Threonine	C/G
4	235-236**	Serine, Lysine	Threonine, Proline	T/A T/C
5	277**	Serine	Arginine	C/G

* indicates aa changes resulting due to the 258 bp fragment (the 3rd - 261st bp of the 2nd exon of the CD14 gene of buffalo).

**indicates aa changes resulting due to the 265 bp fragment (the 587th to 854th nucleotide of the second exon of the CD14 gene of buffalo).

Table 5. Identified SNPs in the CD14 gene of buffalo include 6 variants.

CD14-A	CD14-B	CD14-C	CD14-D	CD14-E	CD14-F	SNPs identified	Nucleotide Position	Synm/ Non-synm
C	A	C	C	C	C	C/A	618	Synm
C	C	G	G	G	C	C/G	627	Non-synm
G	T	G	G	G	G	G/T	630	Non-synm
T	T	T	C	T	T	C/T	644	Non-synm
C	C	C	T	C	C	C/T	650	Non-synm
G	G	G	A	G	G	G/A	651	Non-synm
A	A	A	A	G	A	A/G	656	Non-synm
A	A	G	A	A	A	A/G	674	Non-synm
G	C	G	G	G	G	G/C	679	Non-synm
C	C	C	G	C	C	C/G	693	Synm
G	A	C	G	G	G	G/A/C	702	Non-synm
G	A	A	A	A	A	A/G/T	703	Non-synm
C	C	A	C	C	C	C/A	704	Non-synm
G	T	G	G	G	G	G/T	732	Synm
A	G	G	G	G	G	G/A	736	Non-synm
G	C	C	C	C	C	C/G	737	Non-synm
A	A	A	C	A	A	A/C	746	Non-synm
A	A	A	C	A	A	A/C	754	Non-synm
G	G	G	G	G	C	G/C	755	Non-synm
T	T	T	A	T	C	T/A/C	764	Non-synm
C	C	C	C	C	G	C/G	765	Non-synm
G	G	G	A	G	A	G/A	767	Non-synm
A	A	A	G	A	C	A/G/C	770	Non-synm
G	G	G	C	G	C	G/C	797	Non-synm
G	G	G	G	G	C	G/C	799	Non-synm
C	C	C	C	C	A	C/A	800	Non-synm
T	T	T	T	T	G	T/G	801	Non-synm
G	G	G	G	G	A	G/A	811	Non-synm
T	T	T	C	T	C	T/C	812	Non-synm
C	C	C	C	C	A	C/A	813	Non-synm
C	C	C	G	C	C	C/G	817	Non-synm
C	C	C	A	C	C	C/A	819	Non-synm
A	A	A	C	A	A	A/C	820	Non-synm
G	G	G	C	G	G	G/C	821	Non-synm
T	T	T	C	T	T	T/C	822	Non-synm
C	C	C	C	C	T	C/T	824	Non-synm
A	A	A	A	A	G	A/G	825	Non-synm
G	G	G	T	G	G	G/T	830	Non-synm
G	G	G	G	G	G	G/C	831	Non-synm
A	A	A	G	A	A	A/G	838	Non-synm
G	T	T	G	T	T	T/G	844	Non-synm
T	T	T	C	T	T	T/C	847	Non-synm

Synm: Nucleotide substitution leading to synonymous codon change.

Non-synm : Nucleotide substitution leading to non-synonymous codon change.

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*Continued from page 137

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