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Genetic Characterization of Pakistani Goat Breeds through Mitochondrial D-Loop and Cytochrome b Gene Analyses

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Authors' Contribution
HS performed research work and assisted in data anaylsis. MEB was the principal investigator of the project, supervised the study and reviewed it. AN and TH did the sampling, performed lab work and helped in data analysis and paper write-up. MTP helped in data analysis. MM, HU and JK assisted in paper write up, review and editing.

Key words
Cytochrome b, Displacement-loop, Goat breeds, Haplotypes, Pakistan, Phylogenetic tree, Variable sites

ABSTRACT

We analyzed the complete mitochondrial DNA displacement-loop (D-loop) and the cytochrome *b (cyt b)* gene of ten Pakistani domestic goat breeds (*Capra hircus*). The sequence analysis showed 209 variable sites in goat breeds and each haplotype was unique. All the haplotypes were rich in A/T content. Analysis of variable sites revealed 178 transitions and 31 transversions. Out of 178 transitions 81 were A↔G and 97 were C↔T. Of the 31 transversions, 2 were heteromorphic transversion G→T/C and T→G/C, one transversion also showed a pattern of transition. The ratio between transitions to transversion was 8.5:1.5. The phylogenetic analyses and sequence divergence (SD) established two major distinct mt-lineages termed as *A, B*. An A mt-lineage is further branched into three clades A1, A2, A3. This suggested that at least two different strains of wild *Capra* might have been the source of the modern domestic goats. Phylogenetic analysis of all haplotypes of this research work was performed with some reported haplotypes of *Capra hircus* and wild goats. Origin of domestication seemed to be from Europe, Asia and Africa. Our haplotypes have close relationship with Cashmiree breed, inner Mangolia and probably have originated from wild *Capra aegagrus*.

INTRODUCTION

Small ruminants are very important part of livestock production and agricultural systems worldwide particularly in the semi-arid and arid regions. Goats play very vital role in the livelihood of rural peoples and contribute significantly to poverty alleviation and food security among the poor peoples and farmers in many developing countries of Africa and Asia (Leme *et al*., 2014). According to estimates (FAO, 2014) of the total population of 1.0 billion, more than 590 breeds of goats have been characterized on the basis of genetic diversity (Leme *et al*., 2014) Asia in this regard shares more than 58% population of global goat possessing significant genetic diversity. 31% of goat breeds existing globally are from the region of Asia and these breeds of goats have different beneficial properties like ability to produce animal protein by converting the low quality feedstuff, disease resistance and adaptation to extreme environmental conditions (Hoffmann, 2010). Mitochondrial DNA (mtDNA) of higher animals is a double stranded, closed circular molecule of 16-23 kb, and is regarded as one of the key regulators of metabolism and apoptosis (MacHugh and Bradley, 2001). Mitochondria are the only organelles outside nucleus

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containing genes. Due to its unique properties of having low molecular weight, stability, simple structure, and maternal inheritance, mitochondria best suited for studying animal origins, evolutionary process, and differentiation (Chen *et al*., 2006). mtDNA of most mammals possess 37 genes and a non-coding sequence of variable length, called D-loop or control region. The 37 genes contain 22 transfer RNA (tRNA) genes, 13 protein-coding genes, and two ribosomal RNA (rRNA) genes. The 13 protein- coding genes are cytochrome c oxidase subunits, cytochrome b (*Cytb*), ATP synthase subunits *ATPase6* and *ATPase8*, and seven subunits of NADH dehydrogenase. The two rRNA genes are *12s rRNA* and *16s rRNA*. D-loop region and Mitochondrial *Cyt-b* gene have provided significant insights into domestication, past migration history and characterization of various phylogenetic relations (Ahmed *et al*., 2017; Goldstein and Pollock, 1997). *Cytochrome* b gene of mitochondria has a moderate evolutionary rate and a clear evolutionary pattern that makes it suitable for the studies regarding phylogenetic evolution at both inter and intra-species levels (Lestari *et al*., 2018). Exploration of genetic diversity at molecular level is very useful counterpart for the evaluation of production systems and phenotypes. It provides valuable information about breed history, guides about the development of breeds and also helps to take decision about conservation (Ajmone-Marsan *et al*., 2014; Diwedi *et al*., 2020). Molecular data can be particularly helpful in identifying potential conservation gaps when knowledge about phenotype is inadequate (Dixit *et al*., 2010; Oetting and King, 1999). During this study, it was aimed at molecularly characterizing Pakistani goat breeds so as to identify novel SNPs of in mitochondrial D- loop and cytochrome *b* region, to determine the specie differences of Pakistani goat and to study the evolutionary characterization of Pakistani goat breeds.

MATERIALS AND METHODS

Breeds selection

Ten different breeds of goats with particular phenotypic features (Table I) throughout Pakistan were selected from Government livestock farms and breeding tracts and their blood samples were collected in 15 ml tubes containing EDTA.

Amplification, purification and DNA sequencing

DNA was extracted by using organic method (Sambrook and Russell, 2001). For a total of 2622bp region, starting from Cytochrome b and ending at D-loop, including the start 85bp of mitochondria, four sets of

Table I. Sampling areas of different goat breeds.

S. No	Breed	Sample size	Location.	Province
1	Barbari	05	Baram baran Sibi	Balochistan
2	Beetal	05	Small Ruminant Patoki	Punjab
3	Pahari hairy	05	Quetta	Balochistan
4	Kamori	05	Kamori goat farm, Khuda Abad	Sindh
5	Damani	05	D.I Khan Near Gomal University	Khyber Pakhtunkhwa
6	Khurasani	05	Swat valley	Khyber Pakhtunkhwa
7	L.hairy	05	BLPRI, Kherimurat, Attok	Khyber Pakhtunkhwa
8	Teddy	05	Chak Katora Hasil Pur	Punjab
9	Lehri	05	Mithri bolan/Village Aziz Raisani	Balochistan
10	Nachi	05	Rakh Khairwala(Layyah)	Punjab

Table II. Sequence of primers used for amplification of 2622 bp region of mitochondrial DNA, starting from Cytochrome b and ending at D-loop, including the start 85 bp of mitochondria.

Set of primers	Sequence of primers 5`→3`	Annealing temp	Product size (bP)
Set 1	F: CCAATGATATGAAAAACCATCG R: TCTTAGGCGCCATGCTACTA	57 °C	752
Set 2	F: ACAGGAATTCCATCAGACACAG R: AACCAGAAAAGGAGAATAGCCA	57 °C	575
Set 3	F: AAGCCATAGCCTCACTATCAGC R: ACATCTGGTTCTTTCTTCAGG	50 °C	830
Set 4	F: GATCACGAGCTTGTTGACCA R: TAAACACATAGGTTTGGTCCCAG	50 °C	659

primers (Table II) were designed using primer 3 software in overlapping manner (Rozen and Skaletsky, 2000). For PCR reaction to perform, 150-200 ng genomic DNA, 240 µM of each dNTP, 1 unit of Taq DNA polymerase, 4.5 pmol of each primer and 1x Taq reaction buffer were used in 25 µL reaction volume. The reaction was carried out through 35 cycles that consisted of denaturation at 95°C for 30 second, annealing at 57/50°C for 35 sec and extension at 72°C for 30 sec. During the first cycle, denaturation was done at 95°C for 5 min while the final extension was done at 72°C for 10 min. Gel electrophoresis of the PCR product was done on 1.5% agarose gel containing gel red for visualization. The amplified products were processed using ethanol precipitation method and bidirectionally sequenced through direct Sanger method (Sanger *et al*., 1977).

Sequence analysis and polymorphism detection

The sequences were blast against reference sequence by using Chromas software version 2.2.10 (Tatusova and Madden, 1999). Single nucleotide polymorphic sites were detected from aligned sequences. Any change in the DNA sequence was confirmed by sequencing both sense and antisense strands. Analysis of the sequences was done with the help of appropriate software. Phylogenetic tree was prepared by using Mega 3.1 (Kumar *et al*., 2004).

RESULTS

This study, focused on molecularly characterizing Pakistani goat breeds by identify SNPs in mitochondrial D- loop and Cytochrome *b* region, determining the specie differences of Pakistani goat and analyzing evolutionary characterization of Pakistani goat breeds. Cytochrome b and D-loop region lie in 14151-15290bp and 15429-16640bp, respectively. A total of 2622bp region was selected for sequencing that consisted of 138bp section lying between cytochrome b and D- loop, the region from 14103 to16640bp and 85bp section tRNA post D-loop. Two hundred and nine variable sites were observed. Haplotype diversity showed each haplotype was unique. All the haplotypes were rich in A/T content (Table II). The variable sites revealed 178 transitions and 31 transversions (Supplementary Table I). Of 178 transitions, 81 were A↔G and 97 were C↔T (Supplementary Table I). Of the total 31 transversions, 02 were heteromorphic transversions G→T/C and T→G/C (Supplementary Table I). One transversion also showed a pattern of transition. The ratio between transition to transversion was 8.5:1.5.

A phylogenetic tree constructed from the 50 haplotypes of Pakistani Goat (Cytochrome b and mtD-loop) (Figs. 1 and 2) showed 2 major clad with branching and rebranching pattern. The two maternal lineages were A and B. Lineage A had three mini clades A1, A2, A3. A comparison of haplotypes of the present study with previously reported haplotypes showed that (Fig. 3) that one haplotype seemed to be evolved from Europe and others from India and Africa. This topology was further confirmed by constructing Parsimony tree (Fig. 3). Neighbor joining tree showed many nodes and branches in phylogenetic tree (Fig. 1). This type of distribution indicated the migration of animals for trade purpose and their DNA shuffling due to breeding between different breeds. NJ Phylogenetic tree (Fig. 1) and maximum parsimony tree of goat (Fig. 3) showed intermingled results, no breed differentiation. Domestic goat had very close relation with wild *Capra aegagrus.* There were 41 insertions and 9 deletions observed in goat with reference to sheep (AF010406.1).

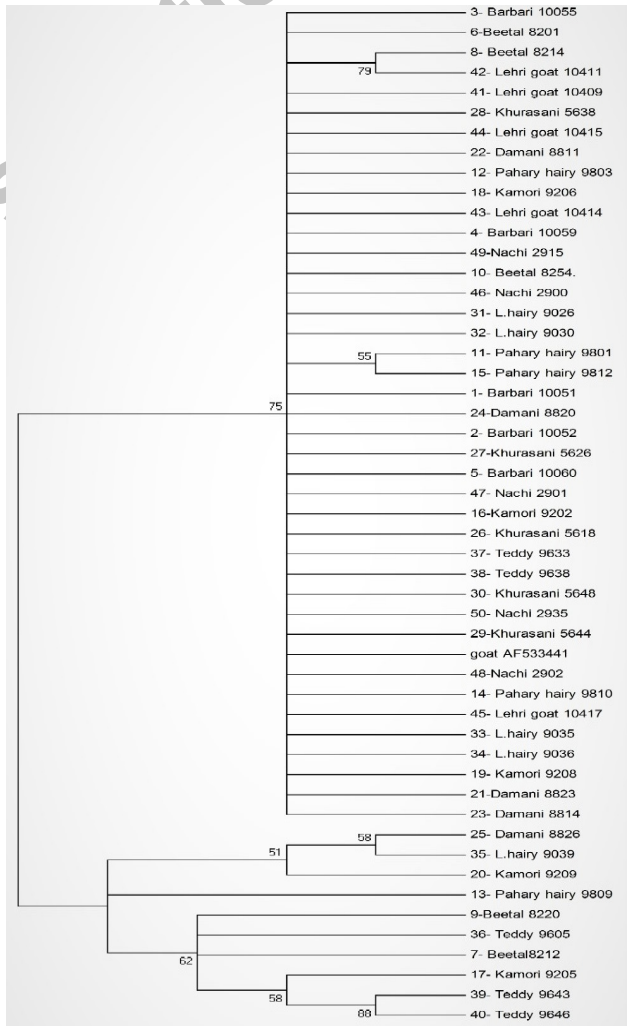

Fig. 1. Neighbour phylogenetic tree (Mega 4.1, Goat).

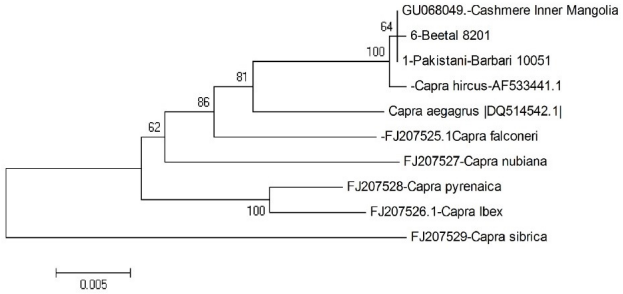

Fig. 2. Comparison of haplotypes of goat in this research work with Capra family.

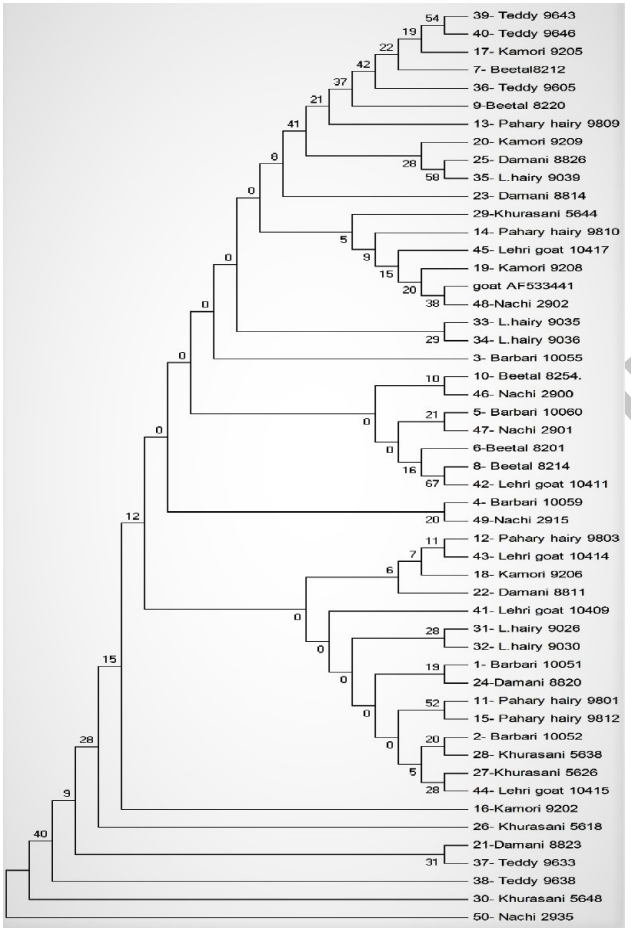

Fig. 3. Maximum parsimony tree (Goat breeds Mega 4.1 goat).

DISCUSSION

The present study was planned to explore D-loop and cytochrome b of Pakistani goat breeds for better understanding the determination of genetic diversity and breeds/species differentiation, and the future plan of breed selection and conservation accordingly. The haplotypes combined region of cytochrome b and D-loop were unique. The existence of mutations in *cytochrome b* were highly associated with D-loop region. There were 209 variable sites found in a 1190bp sequence of *Cyt b* gene, much higher than what was reported previously (Kamalakkannan *et al*., 2018). Neighbour joining phylogenetic tree showed domestic goat with at least two maternal lineages, A and B. Teddy breed came at the bottom of A lineage. Some other breeds like Kamori, Beetal, Pahary hairy and Damani (10 haplotypes) were also included in lineage A. however other group B has all 10 breeds (40 haplotypes). Earlier studies on mtDNA control region of 30 Pakistani domestic goats had reported 22 were sequenced. Twenty-two new haplotypes with two distinct clusters in mt-lineage A, into A1 and A2 (Sultana and Mannen, 2004). Similarly, McNulty *et al*. (2004) reported two types of maternal origins from lineage A and lineage B in Chinese goats (Chen *et al*., 2006; McNulty *et al*., 2004). The findings of the present study did agree with previous reports in terms of haplogrouping. Wang *et al*. (2008) studied 107 entities, belonging to 7 breeds of Chinese goat and founded haplogroup C group in the inner Mongolia and Taihang, and haplogroup D in breeds of inner Mongolia, Taihang, Jining (Wang *et al*., 2008). On analyzing 6 subpopulations of goats, Zhao *et al*. (2011) observed 21 insertion mutations and 29 transversions mutation in the D-loop of mitochondria, showing Cyt b to be more conserved compared to non-coding region of D-loop (Zhao *et al*., 2011). This study, on the basis of phylogenetic trees, showed that breeds were intermixed, no clear significant difference was found between breeds, most probably because of migration flow and gene flow, climatic changes and hybridization pattern. To understand the origin of goat breeds, different haplotypes reported globally were compared haplotypes of the present findings. The phylogenetic trees revealed that one haplotype originated from Europe while the 2nd cluster had close association with breeds of India and Namibia. The main reason behind this association could be the migration of animals for trade purposes and their DNA shuffling due to breeding between different breeds accordingly. Studies showed that the domestic goat (*C. hircus*), the bezoar (*Capra aegagrus*), and the markhor (*C. falconeri*) belong to one clade, plausibly because of introgression between ancestral taxa before colonization to Europe and East Africa (Pidancier *et al*., 2006; Zeder *et al*., 2006). Mitochondrial and nuclear DNA analysis revealed that domestic goat *Capra hircus* originated and domesticated from the bezoar *Capra aegagrus* (Pidancier *et al*., 2006; Takada *et al*., 1997; Zeder, 2005). Phylogenetic tree showed that *Capra hircus* and new haplotypes of the present study were very close to domestic goat, strong

association with haplotype of Cashmeree goat and bezoar *Capra aegagrus*. The findings of this work hence, support the previous reports but still needs to compare molecularly with Pakistani sheep breeds to have better understanding for conservation, adaptability to heat stress conditions and commercialization.

CONCLUSION

We analyzed the complete mitochondrial DNA Displacement-loop (D-loop) and the Cytochrome *b (cyt b)* gene of ten Pakistani domestic goat breeds (*Capra hircus*). The sequence analysis showed 209 variable sites in goat breeds and each haplotype was unique. All the haplotypes were rich in A/T content. Analysis of variable sites revealed 178 transitions and 31 transversions. From the results it has been concluded that Pakistani goats are purely domestic and has a close relation with Capra aegagrus and *Capra sibrica.*

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IRB approval and ethical approval

Current study was approved by Ethical committee of Virtual University ERB (vide letter no. 215/mb/vu dated 09/02/2021).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20230424060425

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Table 1: Polymorphic sites detected in cytochrome b and the D-loop region of goat.

Sr. No	SNPs Position	SNP Nucleotide	Change with	Transition / Transversion
1	14151	A	G	Transition
2	14158	A	G	Transition
3	14187	A	G	Transition
4	14192	A	C	Transversion
5	14193	A	C	Transversion
6	14199	G	A	Transition
7	14217	A	G	Transition
8	14255	C	T	Transition
9	14256	C	G	Transversion
10	14260	T	G	Transversion
11	14282	A	G	Transition
12	14286	C	T	Transition
13	14288	G	A	Transition
14	14291	A	G	Transition
15	14316	T	C	Transition
16	14324	C	T	Transition
17	14333	A	G	Transition

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Sr. No	SNPs Position	SNP Nucleotide	Change with	Transition / Transversion
18	14336	A	T	Transversion
19	14360	T	C	Transition
20	14442	A	G	Transition
21	14459	T	C	Transition
22	14495	T	C	Transition
23	14498	A	G	Transition
24	14510	G	A	Transition
25	14514	G	A	Transition
26	14516	A	G	Transition
27	14522	G	A	Transition
28	14525	C	T	Transition
29	14547	T	C	Transition
30	14553	T	C	Transition
31	14576	G	A	Transition
32	14585	C	T	Transition
33	14591	T	C	Transition
34	14625	A	G	Transition
35	14630	A	G	Transition
36	14651	G	A	Transition
37	14744	C	T	Transition
38	14750	C	G	Transversion
39	14793	A	G	Transition
40	14818	A	G	Transition
41	14885	C	T	Transiton
42	14963	G	A	Transition
43	14966	G	A	Transition
44	14969	T	C	Transition
45	14981	A	G	Transition
46	14991	C	T	Transition
47	14993	A	T	Transversion
48	15002	C	T	Transition
49	15005	C	T	Transition
50	15008	C	T	Transition
51	15026	A	C	Transversion
52	15033	G	A	Transition
53	15048	A	T	Transversion
54	15051	T	C	Transition
55	15057	C	A	Transversion
56	15060	G	A	Transversion
57	15074	C	T	Transition
58	15080	T	A	Transversion

Continues to next page....

Sr. No	SNPs Position	SNP Nucleotide	Change with	Transition / Transversion
59	15083	A	G	Transition
60	15089	A	G	Transition
61	15113	C	T	Transition
62	15119	C	T	Transition
63	15122	A	G	Transition
64	15134	G	A	Transition
65	15140	A	C	Transversion
66	15143	T	C	Transition
67	15147	T	A	Transversion
68	15170	A	C	Transversion
69	15179	C	T	Transition
70	15185	T	C	Transition
71	15194	T	C	Transition
72	15218	T	C	Transition
73	15236	T	C	Transition
74	15242	A	C	Transversion
75	15253	C	T	Transition
76	15262	C	T	Transition
77	15266	T	C	Transition
78	15268	G	A	Transition
79	15278	T	C	Transition
80	15337	A	G	Transition
81	15429	A	C	Transversion
82	15472	A	C	Transversion
83	15540	C	T	Transition
84	15541	C	T	Transition
85	15552	C	T	Transition
86	15558	A	G	Transition
87	15566	G	A	Transition
88	15596	T	C	Transition
89	15614	C	T	Transition
90	15620	C	T	Transition
91	15629	A	G	Transition
92	15634	G	A	Transition
93	15635	T	C	Transition
94	15660	G	A	Transition
95	15673	T	C	Transition
96	15675	C	T	Transition
97	15676	A	G	Transition
98	15687	T	C	Transition
99	15689	C	T	Transition

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Sr. No	SNPs Position	SNP Nucleotide	Change with	Transition / Transversion
100	15697	G	A	Transition
101	15708	G	A	Transition
102	15726	G	A	Transition
103	15742	C	T	Transition
104	15745	A	G	Transition
105	15747	G	A	Transition
106	15761	G	A	Transition
107	15769	G	A	Transition
108	15795	A	G	Transition
109	15796	T	C	Transition
110	15805	T	C	Transition
111	15808	G	A	Transition
112	15809	T	C	Transition
113	15810	A	G	Transition
114	15811	C	T	Transition
115	15820	T	C	Transition
116	15831	C	T	Transition
117	15833	A	G	Transition
118	15841	G	A	Transition
119	15850	T	C	Transition
120	15860	A	G	Transition
121	15862	C	T	Transition
122	15868	T	C	Transition
123	15871	A	G	Transition
124	15885	G	T/C	Transversion
125	15891	C	T	Transition
126	15894	C	T	Transition
127	15896	G	A	Transition
128	15907	A	G	Transition
129	15909	A	G	Transition
130	15911	G	A	Transition
131	15918	G	A	Transition
132	15928	T	C	Transition
133	15945	T	C	Transition
134	15948	T	C	Transition
135	15962	A	G	Transition
136	15963	T	C	Transition
137	15966	A	G	Transition
138	15967	T	C	Transition
139	15971	A	G	Transition
140	15972	T	C	Transition

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Sr. No	SNPs Position	SNP Nucleotide	Change with	Transition / Transversion
141	15973	T	C	Transition
142	15974	T	C	Transition
143	15975	C	T	Transition
144	15976	T	C	Transition
145	15979	C	T	Transition
146	15980	G	A	Transition
147	15981	G	A	Transition
148	15982	C	T	Transition
149	15990	A	G	Transition
150	15992	T	G/C	Transversion
151	15998	T	C	Transition
152	15999	T	C	Transition
153	16000	A	G	Transition
154	16001	C	T	Transition
155	16004	C	T	Transition
156	16007	A	G	Transition
157	16008	T	C	Transition
158	16009	T	C	Transition
159	16017	G	A	Transition
160	16020	A	G	Transition
161	16025	G	A	Transition
162	16026	G	A	Transition
163	16035	C	T	Transition
164	16036	A	G	Transition
165	16038	T	C	Transition
166	16040	T	C	Transition
167	16041	T	C	Transition
168	16043	C	T	Transition
169	16046	A	G	Transition
170	16051	C	A	Transition
171	16052	G	A	Transition
172	16064	C	T	Transition
173	16069	T	G	Transversion
174	16071	A	G	Transition
175	16081	T	C	Transition
176	16112	T	C	Transition
177	16143	A	T	Transversion
178	16147	A	C	Transversion
179	16148	C	T	Transition
180	16149	C	T	Transition
181	16158	C	A	Transversion

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Sr. No	SNPs Position	SNP Nucleotide	Change with	Transition / Transversion
182	16186	T	G	Transversion
183	16205	A	T	Transversion
184	16211	C	T	Transition
185	16230	C	T	Transition
186	16263	A	G	Transition
187	16288	A	G	Transition
188	16323	C	G	Transversion
189	16361	C	T	Transition
190	16367	G	C	Transversion
191	16374	A	G	Transition
192	16423	C	G	Transversion
193	16424	A	G	Transition
194	16433	A	G	Transition
195	15438	T	C	Transition
196	16445	C	T	Transition
197	16456	T	C	Transition
198	16458	T	C	Transition
199	16464	G	C	Transversion
200	16475	T	C	Transition
201	16483	C	T	Transition
202	16493	T	C	Transition
203	16512	G	A	Transition
204	16518	T	C	Transition
205	16542	T	C	Transition
206	16614	G	A	Transition
207	16615	T	C	Transition
208	16637	A	G	Transition
209	16639	T	A	Transversion