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Detection of Polymorphism and *In Silico* Characterization of Fecundity Gene (MTNR1A) in Beetal Goats of Punjab, India

Priya Sharma*, Mandeep Sangla, R.S.Sethi and C.S.Mukopadhyay
GADVASU, Ludhiana, Punjab
**Email: priya21sharma9@gmail.com*

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ABSTRACT

Melatonin Receptor 1A regulates seasonal reproduction and circadian rhythms in goats and sheep. Limited reports are available on the polymorphism of the MTNR1A gene, especially in indigenous goats. This study investigated genetic polymorphism and the evolutionary perspectives of the MTNR1A gene in Beetal goats from Punjab. Blood samples of 35 female Beetal goats were collected, and DNA was isolated from PBMCs using the PCI method. PCR amplified the exon 2 region of the MTNR1A gene (856 bp). The samples were screened by PCR-RFLP by digestion with the restriction enzyme, *RsaI*. No SNPs were detected at all four cleavage sites of the *RsaI* enzyme in the 856 bp amplicon of the MTNR1A gene. Phylogenetic analysis of the MTNR1A gene using MEGA 11 indicated that breeds of goats were evolutionarily divergent from ruminant and non-ruminant species. Selection pressure analysis showed that the MTNR1A gene has evolved under neutral selection between species. Secondary and tertiary structure predictions using Psipred and Swiss Model tools, respectively, showed a combination of α- helix, strand, and coil structures for the MTNR1A protein. The Ramachandran plot analysis using the Molprobity indicated that 93.4% and 97.9% of all amino acid residues of the MTNR1A protein in Beetal goats were in favoured and allowed regions, respectively, indicating a correct prediction of conformation. Further research in a larger sample is warranted to explore informative SNPs in the MTNR1A gene.

Keywords: MTNR1A, polymorphism, PCR-RFLP, phylogenetic analysis

INTRODUCTION

Goat rearing demands only minimal investment and does not necessitate any specific or specialized feed, and thus it has gained importance in the livelihoods of marginal farmers and landless labourers (Kujur *et al*. 2015). Beetal goat is the native breed of Punjab, a northern state in India (Tantia *et al*., 2001), known for its higher milk production and thus is used in crossbreeding programs aimed at improving low-productive local goats (Sharma *et al*., 2016). The primary challenges in enhancing genetic gain for reproductive traits in goats are the low heritability and the significant impact of epigenetic factors (Abdolahi *et al*., 2019). Thus, animal breeders investigate marker genes linked to significant impacts on reproductive traits. These candidate genes have the potential to increase the number of offspring per conception and can be useful in carrying out marker-assisted selection (MAS) to enhance reproductive traits (Pan *et al*., 2015). Melatonin regulates circadian rhythms and seasonal reproduction (Chu *et al*., 2007). It functions through a receptor-mediated pathway at the ovarian level, influencing various processes such as oocyte maturation, ovulation, steroidogenesis, follicular growth, and luteinization (Soni *et al*., 2020). There are two distinct subtypes of melatonin receptors: Melatonin Receptor 1A (MTNR1A/MT1) and Melatonin Receptor 1B (MTNR1B/MT2) in mammals (Agaoglu *et al*., 2015). The MTNR1A gene encodes the MT1 receptor. *The* MTNR1A gene has been identified as a fecundity gene, which is known for its correlation with seasonal reproductive activity in goats and sheep (Pulinas *et al*., 2022). Hence, this study aimed to explore the evolutionary perspectives of the MTNR1A gene and investigate genetic polymorphism in Beetal goats from Punjab.

MATERIALS AND METHODS

Animal Selection: The Institutional Animal Ethics Committee (IAEC) approved the use of animals in this study. Blood samples from female Beetal goats (n=35) with at least parity one were collected from the Directorate of Livestock Farms (DLF) of the University.

Genomic DNA Isolation: Around 5 ml of peripheral blood was carefully collected in tubes containing 0.5 ml EDTA from the jugular vein of Beetal goats. The genomic DNA was isolated using the Phenol-Chloroform-Isoamyl alcohol (PCI) extraction procedure (Green and Sambrook, 2012).

Primer Sequences: Reported primers (Abdolahi *et al*., 2019) specific to the exon 2 region of the MTNR1A gene (Table I) were chosen for the study:

Table I: Primer sequences for the MTNR1A gene

Primer of MTNR1A gene	Primer Sequence (5'-3')	Primer Length (bp)	Product Size (bp)
Forward	GCCTGGCAGTTGCAGACCTG	20	856
Reverse	CATTTTTAAACGGAGTCCACC	21	

Amplification of MTNR1A Gene: The final reaction volume for the polymerase chain reaction was 25µl. The reaction mixture consists of GoTaq® Colorless master mix (Promega, 12.5µl), forward and reverse primers (10pmol/µl), a DNA template, and nuclease-free water. The temperature conditions for initial denaturation, denaturation, annealing, elongation, and final extension were 94°C (5 min), 94°C (30 sec), 61°C (30 sec), 72°C (30 sec), and 72°C (10 min). The amplification of the target region of the MTNR1A gene was checked by agarose gel electrophoresis. For confirmation, one representative sample of the MTNR1A gene (856 bp) was submitted for Sanger sequencing to GeneSpec Labs Pvt. Ltd., Kerala.

PCR-Restriction Fragment Length Polymorphism (RFLP): 7.5µl of PCR amplicon was digested using *RsaI enzyme* (0.5 µl) and the tubes were kept for overnight incubation at 37°C followed by a deactivation step at 65°C for 20 minutes. The R.E. digested fragments were resolved in 2% agarose gel.

In-silico Analysis of MTNR1A Gene: *Homology Search*: Following sequencing, the obtained partial cds of the MTNR1A gene (753bp) in Beetal goat was subjected to BLASTn analysis (Altschul *et al.*, 1990). MTNR1A gene variant nucleotide sequences belonging to divergent species available at the NCBI database were selected based on higher per cent identity and E-value ($<10^{-5}$).

Phylogenetic Tree Construction: MEGA 11 software (Tamura *et al.*, 2021) was used for the phylogenetic tree construction. Models with the lowest BIC and AICc scores are considered to describe the substitution pattern best (Nei and Kumar, 2000). The maximum likelihood (ML) method was used for the construction of the phylogenetic tree. The reliability of the branching patterns in the tree was confirmed by 500 bootstrap replications.

Selection Pressure Analysis: The number of synonymous (dS) and nonsynonymous substitutions (dN) per synonymous and non-synonymous sites, respectively, was used to calculate the test statistic (dN-dS) along with the probability of rejecting the null hypothesis that the codons have evolved through neutral selection (dN = dS) (Singh *et al*., 2015).

Structure Prediction and Validation: The nucleotide sequence of the *MTN1A* gene in Beetal goat was translated to an amino acid sequence and was subjected to secondary and tertiary protein structure prediction by using online tools, Psipred (http://bioinf.cs.ucl.ac.uk/psipred/) and Swiss model (https://swissmodel.expasy.org/), respectively. Further, the predicted tertiary structure was validated through Ramachandran's plot analysis carried out by Molprobity (http://molprobity.biochem.duke.edu/).

RESULTS AND DISCUSSION

PCR-RFLP: Amplified PCR amplicon (856 bp) corresponding to the exon 2 region of the MTNR1A gene was digested with *RsaI*restriction enzyme. It recognizes the site 5’-GT˅AC-3’. Enzyme digestion with *RsaI evidenced* four cleavage sites at positions 29, 306, 329, and 740 in comparison to the reference sequence (Accession no. AB716764.1) and hence, production of five bands of 39bp, 267bp, 23bp, 411bp, and 116bp. PCR-RFLP gave three observable DNA fragments, measuring 267bp, 411bp, and 116bp. The other two bands, 39bp and 23bp, were not visible due to a lack of resolution because of their small size (Figure 1). Hence, it was concluded that the MTNR1A gene is monomorphic at all four loci of restriction enzyme *RsaI*in the given number of samples. The same was validated through Sanger sequencing. After visualizing the chromatogram files with the help of Finch TV software (https://digitalworldbiology.com/FinchTV), again, no SNPs were detected at all four restriction sites of *RsaI* present in the PCR amplicon of the MTNR1A gene. However, many authors have reported the existence of genetic polymorphism in the MTNR1A gene in different goat breeds as detected by PCR-RFLP analysis carried out with the *RsaI* restriction enzyme. Chu *et al*. (2007) have reported *RsaI* polymorphism at position G52A in seasonal oestrous breeds (Jintang Black, Chuandong White, Boer, Guizhou White, Gulin Ma, Banjiao, and Dazu Black) and year-round oestrous breeds (Boer goats). However, the *RsaI* locus of the cleavage site (G52A) was found to be monomorphic in Jining Grey goats, which is in agreement with our study conducted on Beetal goats. The same *RsaI* polymorphism at position G52A has also been reported in Honamli and Hair goats raised in the Western Mediterranean region of Turkey (Agaoglu *et al.*, 2015), and Kacang and Peranakan Ottawa goat populations raised in Indonesia (Dagong *et al.*, 2019). The findings of these studies conducted on different goats, in which the researchers have indicated the presence of *RsaI* polymorphism in the exon 2 region of the MTNR1A gene, are not in agreement with the results obtained for the female Beetal goats in our study, which suggests the monomorphic nature of all *RsaI’*s loci in the targetregion of *the* MTNR1A gene. However, as the sample size is small, this preliminary study is recommended to be validated on a large number of samples. Further investigations are also required to identify and locate other polymorphic sites within the MTNR1A gene that may influence litter size and seasonal reproduction in Beetal goats.

In-silico Analysis of MTNR1A Gene (Homology search)*:* A total of 36 MTNR1A gene variant nucleotide sequences (mRNA/cds) belonging to divergent species available at the NCBI database were selected based on higher per cent identity and E-value ($<10^{-5}$) (Table II).

Phylogenetic Tree Construction: The input 37 nucleotide sequences were used for phylogenetic tree construction using the maximum likelihood method with 500 bootstrap resampling. The T92+G (Tamura 3-parameter + Gamma distributed) model was identified as the most suitable evolutionary model. The formation of distinct clades reflects the evolutionary differences between the sequences. The phylogenetic tree depicted that the breeds of goats were evolutionarily divergent from the breeds of ruminants and non-ruminants. Phylogeny also indicated that *Hemicordylus capensis* (Lizard) was the most distant from the Beetal goat (Figure 2).

Selection Pressure Analysis: The results indicated the acceptance of the null hypothesis of strict neutrality (p-value >0.05), which means that the MTNR1A gene sequence within the species has primarily undergone neutral selection, except between the breed *San Clemente* (p-value = 0.009).

Secondary and Tertiary Structure Prediction: Secondary structure prediction for the MTNR1A protein in Beetal goat using Psipred v3.0 revealed a combination of α- α-helix, strand, and coil structures, whereas the tertiary protein structure analysis through Swiss Model and visualization in RasMol software (http://www.openrasmol.org/) showed β-pleated sheets, α- helix, and loop structures in three-dimensional space (Figure 3). Ramachandran plot analysis by Molprobity revealed that more than 90% of the residues (225/241 and 236/241) fell in the favoured and allowed regions, respectively, indicating a correct prediction of conformation (Figure 4). However, a few legitimate outliers in the plot are often found. For the predicted structure of the MTNR1A protein of the Beetal goat, there were five outliers (phi, psi): 7 Tyr (-131.9, -66.5); 145 Val (73.9, 154.1); 149 Val (-85.7, -141.6); 151 Pro (-111.3, 7.8) and 154 Lys (108.2, -38.7).

SUMMARY

The MTNR1A gene in female Beetal goats was found to be monomorphic for all four *RsaI* restriction sites in this study. However, as the sample size is small, this preliminary study is recommended to be validated on a larger number of samples. The phylogenetic analysis of the MTNR1A gene sequence indicated that breeds of goats were evolutionarily divergent from the breeds of ruminants and non-ruminants. This gene has also undergone neutral selection pressure between divergent species. According to the Ramachandran plot analysis, the predicted three-dimensional structure of the MTNR1A protein in Beetal goat was conformationally correct.

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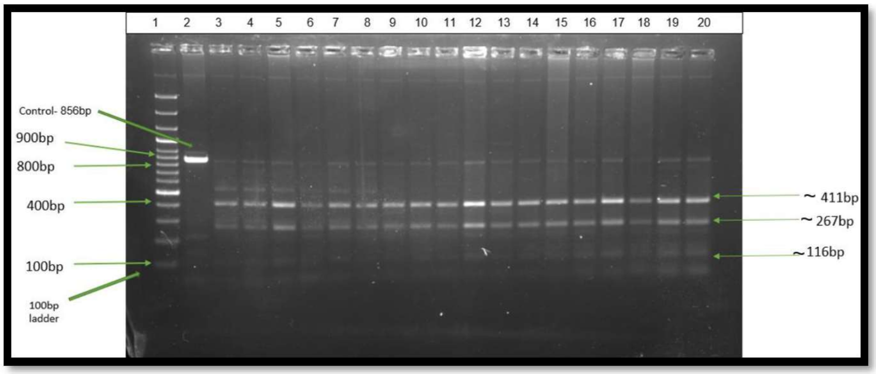


Figure 1: RFLP image of MTNR1A gene by *RsaI* restriction enzyme on 2.0% agarose gel(in lane 3-20 are representative PCR products of MTNR1A gene digested with *RsaI enzyme* and lane 2 consists of control)

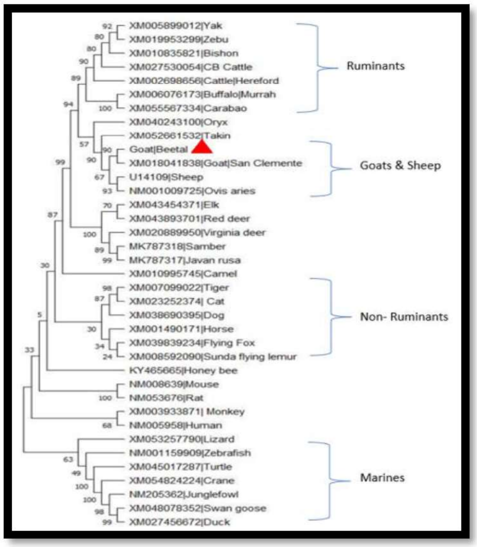


Figure 2: Phylogenetic tree of the *MTNR1A* gene, constructed using MEGA 11 software. The *MTNR1A* gene, in this study, is denoted by a triangle

Figure 3: Prediction of the MTNR1A protein (in Beetal goat) by using Swiss Model visualized under RasMol software

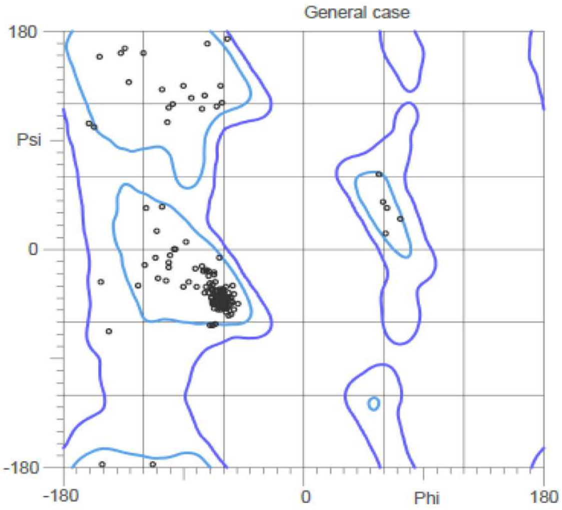


Figure 4: Ramachandran's plot analysis of amino acids present in the MTNR1A protein in Beetal goat using MolProbity

Table II: MTNR1A nucleotide sequences retrieved from NCBI GenBank

Sequence Accession number	Common Name	Scientific name	Breed/ Strain	Country	Sequence type	Sequence length
XM018041838	Goat	*Capra hircus*	San Clemente	-	mRNA	1101
U14109	Sheep	*Ovis aries*	-	-	Complete cds	1149
NM001009725	Sheep	*Ovis aries*	-	-	mRNA	1219
XM040243100	Gemsbok	*Oryx dammah*	-	USA	mRNA	1101
XM027530054	Hybrid cattle	*Bos indicus x Bos taurus*	Angus x Brahman F1 hybrid	_	mRNA	1494
XM002698656	Cattle	*Bos taurus*	Hereford	-	mRNA	1546
XM010835821	Bovine	*Bison bisonbison*	-	-	mRNA	1086
XM006076173	Water buffalo	*Bubalus bubalis*	Murrah	India	mRNA	1560
XM043893701	Red deer	*Cervus elaphus*	-	-	mRNA	1139

XM020889950	Virginia deer	*Odocoileus virginianus texanus*	-	-	mRNA	1047
XM043454371	Elk	*Cervus canadensis*	-	-	mRNA	1139
MK787318	Samber	*Rusa unicolor*	-	Malaysia	Complete cds	814
MK787317	Javan rusa	*Rusatimorensis*	-	Malaysia	Complete cds	888
XM052661532	Takin	*Budorcastaxicolor*	-	-	mRNA	1101
XM005899012	Wild Yak	*Bos mutus*	-	China	mRNA	1002
XM055567334	Carabao	*Bubalus carabanensis*	-	Philippines	mRNA	1478
XM019953299	Zebu	*Bos indicus*	Nelore	Brazil	mRNA	1185
NM008639	Mouse	*Mus musculus*	C57BL/6	-	mRNA	1606
XM053257790	False girdled Lizard	*Hemicordylus capensis*	-	-	mRNA	850
KY465665	Honey bee	*Apis ceranacerana*	-	-	mRNA, Complete cds	963
XM048078352	Swan goose	*Ansercygnoides*	-	-	mRNA	1132
NM053676	Brown rat	*Rattus norvegicus*	-	-	mRNA	1774
XM038690395	Dog	*Canis lupus familiaris*	Labrador retriever	-	mRNA	4498
XM045017287	Yellow pond turtle	*Mauremysmutica*	-	-	mRNA	2011
NM205362	Junglefowl	*Gallus gallus*	-	-	mRNA	3769
XM039839234	Flying Fox	*Pteropus giganteus*	-	-	mRNA	1213
XM008592090	Sunda flying lemur	*Galeopterus variegatus*	-	Indonesia: West Java	mRNA	969
XM010995745	Camel	*Camelus dromedarius*	-	-	mRNA	2125
XM001490171	Horse	*Equus caballus*	Thoroughbred	-	mRNA	3168
XM007099022	Tiger	*Panthera tigris*	-	-	mRNA	1092
NM001159909	Zebrafish	*Danio rerio*	-	-	mRNA	957
XM003933871	Bolivian squirrel monkey	*Saimiri boliviensisboliviensis*	-	-	mRNA	1256
NM005958	Human	*Homo sapiens*	-	-	mRNA	1289
XM027456672	Mallard, wild duck	*Anas platyrhynchos*	-	-	mRNA	1663
XM054824224	Whooping crane	*Grus americana*	-	-	mRNA	1241
XM023252374	Cat	*Felis catus*	-	-	mRNA	1095

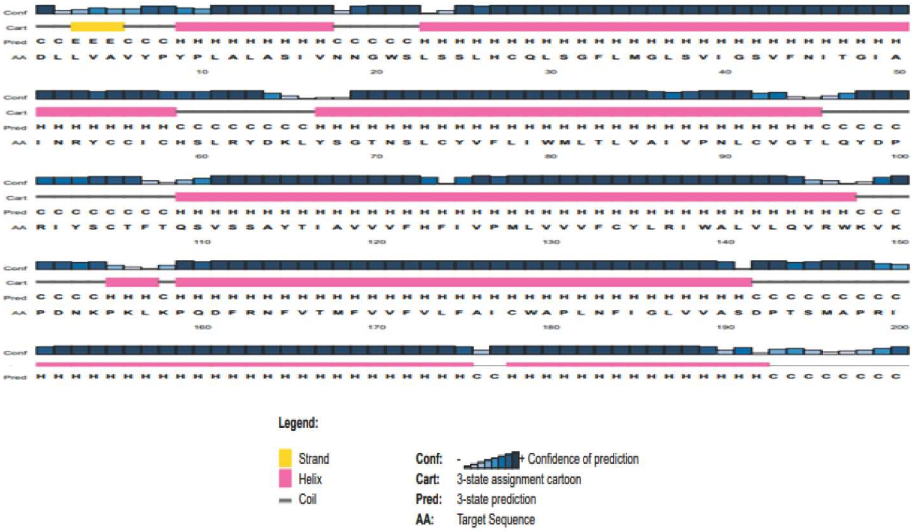
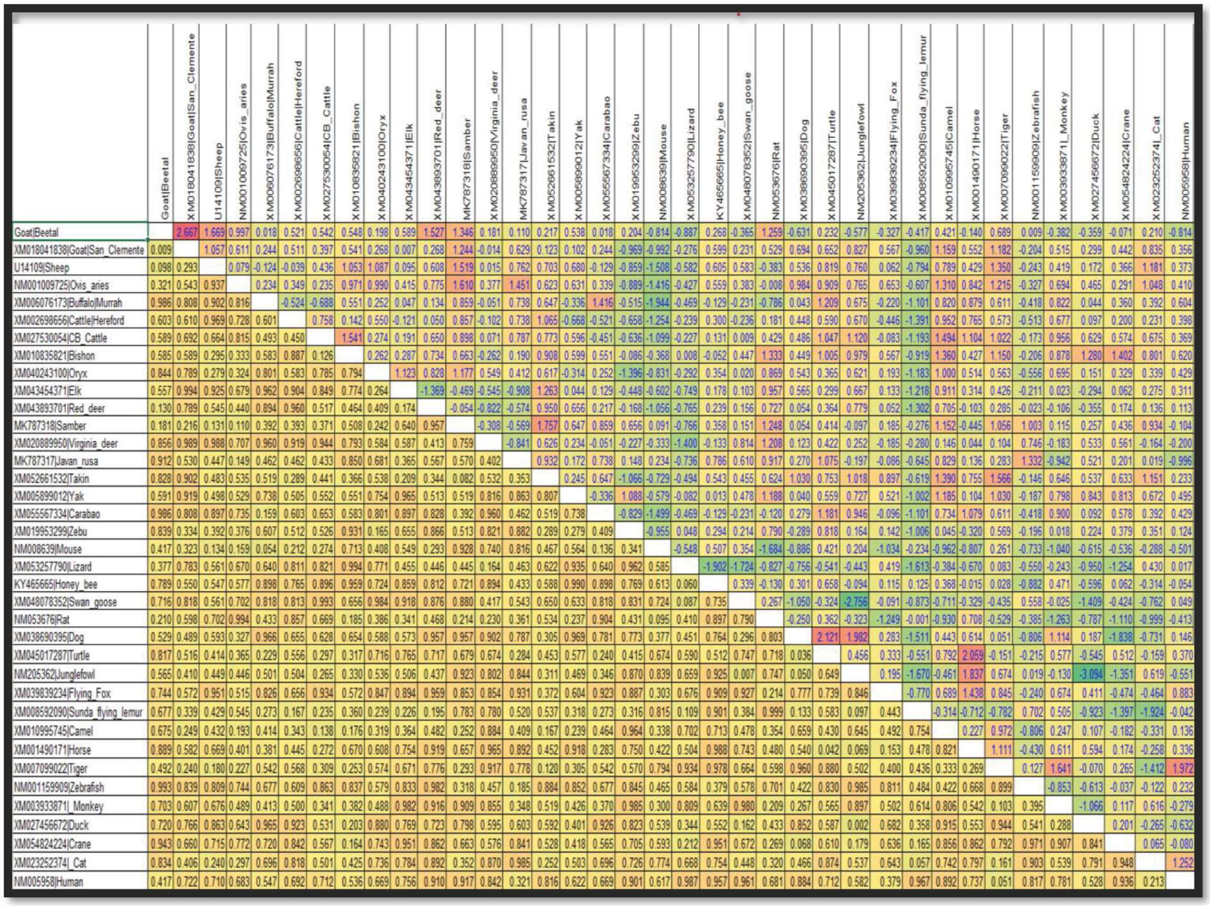


Figure 5: Secondary structure prediction of the MTNR1A protein using Psipred v3.0 software



	Goat|Beetal	XM018041838|Goat|San_Clemente	U14109|Sheep	NM001009725|Ovis_aries	XM006076173|Buffalo|Murrah	XM002698656|Cattle|Hereford	XM027530054|CB_Cattle	XM010835821|Bishon	XM040243100|Oryx	XM043454371|Elk	XM043893701|Red_deer	MK787318|Samber	XM020889950|Virginia_deer	MK787317|Javan_rusa	XM052661532|Takin	XM005899012|Yak	XM055567334|Carabao	XM019953299|Zebu	NM008639|Mouse	XM053257790|Lizard	KY465665|Honey_bee	XM048078352|Swan_goose	NM053676|Rat	XM038690395|Dog	XM045017287|Turtle	NM205362|Junglefowl	XM039839234|Flying_Fox	XM008592090|Sunda_flying_lemur	XM010995745|Camel	XM001490171|Horse	XM007099022|Tiger	NM001159909|Zebrafish	XM003933871|_Monkey	XM027456672|Duck	XM054824224|Crane	XM023252374|_Cat	NM005958|Human
Goat|Beetal		2.667	1.669	0.997	0.018	0.521	0.542	0.548	0.198	0.589	1.527	1.346	0.181	0.110	0.217	0.538	0.018	0.204	-0.814	-0.887	0.268	-0.365	1.259	-0.631	0.232	-0.577	-0.327	-0.417	0.421	-0.140	0.689	0.009	-0.382	-0.359	-0.071	0.210	-0.814
XM018041838|Goat|San_Clemente	0.009		1.057	0.611	0.244	0.511	0.397	0.541	0.268	0.007	0.268	1.244	-0.014	0.629	0.123	0.102	0.244	-0.969	-0.992	-0.276	0.599	0.231	0.529	0.694	0.652	0.827	0.567	-0.960	1.159	0.552	1.182	-0.204	0.515	0.299	0.442	0.835	0.356
U14109|Sheep	0.098	0.293		0.079	-0.124	-0.039	0.436	1.053	1.087	0.095	0.608	1.519	0.015	0.762	0.703	0.680	-0.129	-0.859	-1.508	-0.582	0.605	0.583	-0.383	0.536	0.819	0.760	0.062	-0.794	0.789	0.429	1.350	-0.243	0.419	0.172	0.366	1.181	0.373
NM001009725|Ovis_aries	0.321	0.543	0.937		0.234	0.349	0.235	0.971	0.990	0.415	0.775	1.610	0.377	1.451	0.623	0.631	0.339	-0.889	-1.416	-0.427	0.559	0.383	-0.008	0.984	0.909	0.765	0.653	-0.607	1.310	0.842	1.215	-0.327	0.694	0.465	0.291	1.048	0.410
XM006076173|Buffalo|Murrah	0.986	0.808	0.902	0.816		-0.524	-0.688	0.551	0.252	0.047	0.134	0.859	-0.051	0.738	0.647	-0.336	1.416	-0.515	-1.944	-0.469	-0.129	-0.231	-0.786	0.043	1.209	0.675	-0.220	-1.101	0.820	0.879	0.611	-0.418	0.822	0.044	0.360	0.392	0.604
XM002698656|Cattle|Hereford	0.603	0.610	0.969	0.728	0.601		0.758	0.142	0.550	-0.121	0.050	0.857	-0.102	0.738	1.065	-0.668	-0.521	-0.658	-1.254	-0.239	0.300	-0.236	0.181	0.448	0.590	0.670	-0.446	-1.391	0.952	0.765	0.573	-0.513	0.677	0.097	0.200	0.231	0.398
XM027530054|CB_Cattle	0.589	0.692	0.664	0.815	0.493	0.450		1.541	0.274	0.191	0.650	0.898	0.071	0.787	0.773	0.596	-0.451	-0.636	-1.099	-0.227	0.131	0.009	0.429	0.486	1.047	1.120	-0.083	-1.193	1.494	1.104	1.022	-0.173	0.956	0.629	0.574	0.675	0.369
XM010835821|Bishon	0.585	0.589	0.295	0.333	0.583	0.887	0.126		0.262	0.287	0.734	0.663	-0.262	0.190	0.908	0.599	0.551	-0.086	-0.368	0.008	-0.052	0.447	1.333	0.449	1.005	0.979	0.567	-0.919	1.360	0.427	1.150	-0.206	0.878	1.280	1.402	0.801	0.620
XM040243100|Oryx	0.844	0.789	0.279	0.324	0.801	0.583	0.785	0.794		1.123	0.828	1.177	0.549	0.412	0.617	-0.314	0.252	-1.396	-0.831	-0.292	0.354	0.020	0.869	0.543	0.365	0.621	0.193	-1.183	1.000	0.514	0.563	-0.556	0.695	0.151	0.329	0.339	0.429
XM043454371|Elk	0.557	0.994	0.925	0.679	0.962	0.904	0.849	0.774	0.264		-1.369	-0.469	-0.545	-0.908	1.263	0.044	0.129	-0.448	-0.602	-0.749	0.178	0.103	0.957	0.565	0.299	0.667	0.133	-1.218	0.911	0.314	0.426	-0.211	0.023	-0.294	0.062	0.275	0.311
XM043893701|Red_deer	0.130	0.789	0.545	0.440	0.894	0.960	0.517	0.464	0.409	0.174		-0.054	-0.822	-0.574	0.950	0.656	0.217	-0.168	-1.056	-0.765	0.239	0.156	0.727	0.054	0.364	0.779	0.052	-1.302	0.705	-0.103	0.285	-0.023	-0.106	-0.355	0.174	0.136	0.113
MK787318|Samber	0.181	0.216	0.131	0.110	0.392	0.393	0.371	0.508	0.242	0.640	0.957		-0.308	-0.569	1.757	0.647	0.859	0.656	0.091	-0.766	0.358	0.151	1.248	0.054	0.414	-0.097	0.185	-0.276	1.152	-0.445	1.056	1.003	0.115	0.257	0.436	0.934	-0.104
XM020889950|Virginia_deer	0.856	0.989	0.988	0.707	0.960	0.919	0.944	0.793	0.584	0.587	0.413	0.759		-0.841	0.626	0.234	-0.051	-0.227	-0.333	-1.400	-0.133	0.814	1.208	0.123	0.422	0.252	-0.185	-0.280	0.146	0.044	0.104	0.746	-0.183	0.533	0.561	-0.164	-0.200
MK787317|Javan_rusa	0.912	0.530	0.447	0.149	0.462	0.462	0.433	0.850	0.681	0.365	0.567	0.570	0.402		0.932	0.172	0.738	0.148	0.234	-0.736	0.786	0.610	0.917	0.270	1.075	-0.197	-0.086	-0.645	0.829	0.136	0.283	1.332	-0.942	0.521	0.201	0.019	-0.996
XM052661532|Takin	0.828	0.902	0.483	0.535	0.519	0.289	0.441	0.366	0.538	0.209	0.344	0.082	0.532	0.353		0.245	0.647	-1.066	-0.729	-0.494	0.543	0.455	0.624	1.030	0.753	1.018	0.897	-0.619	1.390	0.755	1.566	-0.146	0.646	0.537	0.633	1.151	0.233
XM005899012|Yak	0.591	0.919	0.498	0.529	0.738	0.505	0.552	0.551	0.754	0.965	0.513	0.519	0.816	0.863	0.807		-0.336	1.088	-0.579	-0.082	0.013	0.478	1.188	0.040	0.559	0.727	0.521	-1.002	1.185	0.104	1.030	-0.187	0.798	0.843	0.813	0.672	0.495
XM055567334|Carabao	0.986	0.808	0.897	0.735	0.159	0.603	0.653	0.583	0.801	0.897	0.828	0.392	0.960	0.462	0.519	0.738		-0.829	-1.499	-0.469	-0.129	-0.231	-0.120	0.279	1.181	0.946	-0.096	-1.101	0.734	1.079	0.611	-0.418	0.900	0.092	0.578	0.392	0.429
XM019953299|Zebu	0.839	0.334	0.392	0.376	0.607	0.512	0.526	0.931	0.165	0.655	0.866	0.513	0.821	0.882	0.289	0.279	0.409		-0.955	0.048	0.294	0.214	0.790	-0.289	0.818	0.164	0.142	-1.006	0.045	-0.320	0.569	-0.196	0.018	0.224	0.379	0.351	0.124
NM008639|Mouse	0.417	0.323	0.134	0.159	0.054	0.212	0.274	0.713	0.408	0.549	0.293	0.928	0.740	0.816	0.467	0.564	0.136	0.341		-0.548	0.507	0.354	-1.684	-0.886	0.421	0.204	-1.034	-0.234	-0.962	-0.807	0.261	-0.733	-1.040	-0.615	-0.536	-0.288	-0.501
XM053257790|Lizard	0.377	0.783	0.561	0.670	0.640	0.811	0.821	0.994	0.771	0.455	0.446	0.445	0.164	0.463	0.622	0.935	0.640	0.962	0.585		-1.902	-1.724	-0.827	-0.756	-0.541	-0.443	0.419	-1.613	-0.384	-0.670	0.083	-0.550	-0.243	-0.950	-1.254	0.430	0.017
KY465665|Honey_bee	0.789	0.550	0.547	0.577	0.898	0.765	0.896	0.959	0.724	0.859	0.812	0.721	0.894	0.433	0.588	0.990	0.898	0.769	0.613	0.060		0.339	-0.130	0.301	0.658	-0.094	0.115	0.125	0.368	-0.015	0.028	-0.882	0.471	-0.596	0.062	-0.314	-0.054
XM048078352|Swan_goose	0.716	0.818	0.561	0.702	0.818	0.813	0.993	0.656	0.984	0.918	0.876	0.880	0.417	0.543	0.650	0.633	0.818	0.831	0.724	0.087	0.735		0.267	-1.050	-0.324	-2.756	-0.091	-0.873	-0.711	-0.329	-0.435	0.558	-0.025	-1.409	-0.424	-0.762	0.049
NM053676|Rat	0.210	0.598	0.702	0.994	0.433	0.857	0.669	0.185	0.386	0.341	0.468	0.214	0.230	0.361	0.534	0.237	0.904	0.431	0.095	0.410	0.897	0.790		-0.250	0.362	-0.323	-1.249	-0.001	-0.930	0.708	-0.529	-0.385	-1.263	-0.787	-1.110	-0.999	-0.413
XM038690395|Dog	0.529	0.489	0.593	0.327	0.966	0.655	0.628	0.654	0.588	0.573	0.957	0.957	0.902	0.787	0.305	0.969	0.781	0.773	0.377	0.451	0.764	0.296	0.803		2.121	1.982	0.283	-1.511	0.443	0.614	0.051	-0.806	1.114	0.187	-1.838	-0.731	0.146
XM045017287|Turtle	0.817	0.516	0.414	0.365	0.229	0.556	0.297	0.317	0.716	0.765	0.717	0.679	0.674	0.284	0.453	0.577	0.240	0.415	0.674	0.590	0.512	0.747	0.718	0.036		0.456	0.333	-0.551	0.792	2.059	-0.151	-0.215	0.577	-0.545	0.512	-0.159	0.370
NM205362|Junglefowl	0.565	0.410	0.449	0.446	0.501	0.504	0.265	0.330	0.536	0.506	0.437	0.923	0.802	0.844	0.311	0.469	0.346	0.870	0.839	0.659	0.925	0.007	0.747	0.050	0.649		0.195	-1.670	-0.461	1.837	0.674	0.019	-0.130	-3.094	-1.351	0.619	-0.551
XM039839234|Flying_Fox	0.744	0.572	0.951	0.515	0.826	0.656	0.934	0.572	0.847	0.894	0.959	0.853	0.854	0.931	0.372	0.604	0.923	0.887	0.303	0.676	0.909	0.927	0.214	0.777	0.739	0.846		-0.770	0.689	1.438	0.845	-0.240	0.674	0.411	-0.474	-0.464	0.883
XM008592090|Sunda_flying_lemur	0.677	0.339	0.429	0.545	0.273	0.167	0.235	0.360	0.239	0.226	0.195	0.783	0.780	0.520	0.537	0.318	0.273	0.316	0.815	0.109	0.901	0.384	0.999	0.133	0.583	0.097	0.443		-0.314	-0.712	-0.782	0.702	0.505	-0.923	-1.397	-1.924	-0.042
XM010995745|Camel	0.675	0.249	0.432	0.193	0.414	0.343	0.138	0.176	0.319	0.364	0.482	0.252	0.884	0.409	0.167	0.239	0.464	0.964	0.338	0.702	0.713	0.478	0.354	0.659	0.430	0.645	0.492	0.754		0.227	0.972	-0.806	0.247	0.107	-0.182	-0.331	0.136
XM001490171|Horse	0.889	0.582	0.669	0.401	0.381	0.445	0.272	0.670	0.608	0.754	0.919	0.657	0.965	0.892	0.452	0.918	0.283	0.750	0.422	0.504	0.988	0.743	0.480	0.540	0.042	0.069	0.153	0.478	0.821		1.111	-0.430	0.611	0.594	0.174	-0.258	0.336
XM007099022|Tiger	0.492	0.240	0.180	0.227	0.542	0.568	0.309	0.253	0.574	0.671	0.776	0.293	0.917	0.778	0.120	0.305	0.542	0.570	0.794	0.934	0.978	0.664	0.598	0.960	0.880	0.502	0.400	0.436	0.333	0.269		0.127	1.641	-0.070	0.265	-1.412	1.972
NM001159909|Zebrafish	0.993	0.839	0.809	0.744	0.677	0.609	0.863	0.837	0.579	0.833	0.982	0.318	0.457	0.185	0.884	0.852	0.677	0.845	0.465	0.584	0.379	0.578	0.701	0.422	0.830	0.985	0.811	0.484	0.422	0.668	0.899		-0.853	-0.613	-0.037	-0.122	0.232
XM003933871|_Monkey	0.703	0.607	0.676	0.489	0.413	0.500	0.341	0.382	0.488	0.982	0.916	0.909	0.855	0.348	0.519	0.426	0.370	0.985	0.300	0.809	0.639	0.980	0.209	0.267	0.565	0.897	0.502	0.614	0.806	0.542	0.103	0.395		-1.066	0.117	0.616	-0.279
XM027456672|Duck	0.720	0.766	0.863	0.643	0.965	0.923	0.531	0.203	0.880	0.769	0.723	0.798	0.595	0.603	0.592	0.401	0.926	0.823	0.539	0.344	0.552	0.162	0.433	0.852	0.587	0.002	0.682	0.358	0.915	0.553	0.944	0.541	0.288		0.201	-0.265	-0.632
XM054824224|Crane	0.943	0.660	0.715	0.772	0.720	0.842	0.567	0.164	0.743	0.951	0.862	0.663	0.576	0.841	0.528	0.418	0.565	0.705	0.593	0.212	0.951	0.672	0.269	0.068	0.610	0.179	0.636	0.165	0.856	0.862	0.792	0.971	0.907	0.841		0.065	-0.080
XM023252374|_Cat	0.834	0.406	0.240	0.297	0.696	0.818	0.501	0.425	0.736	0.784	0.892	0.352	0.870	0.985	0.252	0.503	0.696	0.726	0.774	0.668	0.754	0.448	0.320	0.466	0.874	0.537	0.643	0.057	0.742	0.797	0.161	0.903	0.539	0.791	0.948		1.252
NM005958|Human	0.417	0.722	0.710	0.683	0.547	0.692	0.712	0.536	0.669	0.756	0.910	0.917	0.842	0.321	0.816	0.622	0.669	0.901	0.617	0.987	0.957	0.961	0.681	0.884	0.712	0.582	0.379	0.967	0.892	0.737	0.051	0.817	0.781	0.528	0.936	0.213	

Figure 6: Neutral selection of MTNR1A gene estimated using MEGA 11 software