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

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Received: August 2024 *172/24* *Accepted: March 2026*

ABSTRACT

Melatonin Receptor 1A regulates seasonal reproduction in goats and sheep. Limited reports are available on the *MTNR1A* gene polymorphism in indigenous goats. This study investigated genetic polymorphism and evolutionary perspectives of the *MTNR1A* gene in Beetal goats from Punjab. DNA was isolated from blood samples of 35 female Beetal goats. Exon 2 region (856 bp) was amplified and analysed by PCR-RFLP with the *RsaI* enzyme. No SNPs were detected at all four cleavage sites. Phylogenetic analysis indicated evolutionary conservation of the *MTNR1A* gene among goats and sheep. The predicted MTNR1A protein structure comprises β-pleated sheets, α- helix, and loops.

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

INTRODUCTION

Beetal goat is the native breed of Punjab (Tantia *et al.,* 2001). It is highly regarded for its milk production capacity and often used in crossbreeding programs aimed at improving the overall characteristics and grading of low-productive local goats (Sharma *et al.,* 2016). The primary challenges in enhancing genetic gain for reproductive traits within goat populations are the low heritability and the significant impact of epigenetic factors (Abdolahi *et al.,* 2019). Consequently, animal breeders are now investigating 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) and Melatonin Receptor 1B (MTNR1B) in mammals (Agaoglu *et al.,* 2015). The *MTNR1A* gene encodes the MTNR1A receptor. It 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 (5ml) from female Beetal goats (n=35) with at least one parity were collected from the Directorate of Livestock Farms (DLF) of the University.

Genomic DNA Isolation: The genomic DNA was isolated by utilising the 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 (Abdolahi *et al.,* 2019)

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 temperature conditions for initial denaturation, denaturation, annealing, elongation, and final extension were 94^{o}C (5 min), 94^{o}C (30 sec), 61^{o}C (30 sec), 72^{o}C (30 sec), and 72^{o}C (10 min), respectively.

PCR-Restriction Fragment Length Polymorphism (RFLP): PCR amplicon was digested using the *RsaI* enzyme, and the tubes were kept for overnight incubation at 37^{o}C followed by a deactivation step at 65^{o}C for 20 minutes.

In-silico Analysis of MTNR1A Gene

***Homology Search*:** One representative PCR amplicon of the *MTNR1A* gene (856 bp) was sent for Sanger sequencing (GeneSpec Labs Pvt. Ltd., Kerala). The obtained partial cds was subjected to BLASTn analysis (Altschul *et al.,* 1990). *MTNR1A* gene variant nucleotide sequences belonging to divergent species were downloaded.

***Phylogenetic Tree Construction*:** MEGA 11 software (Tamura *et al.,* 2021) was used for the phylogenetic analysis. The tree was constructed using the maximum likelihood (ML) method, and the reliability of the branching patterns 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).

Structure Prediction and Validation: Secondary and tertiary protein structure prediction for the *MTN1A* gene in Beetal goat was done by using online tools, Psipred and Swiss model, respectively, and validated through Ramachandran's plot analysis carried out by Molprobity.

RESULTS AND DISCUSSION

PCR-RFLP

RsaI recognises the site 5'-GT$^{\blacktriangledown}$AC-3'. Enzyme digestion of PCR amplicon (856 bp) 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. However, PCR-RFLP yielded three observable DNA fragments, measuring 267 bp, 411 bp, and 116 bp. The other two bands, 39 bp and 23 bp, were not visible due to their small size and limited resolution (Figure 1). Hence, it was concluded that the *MTNR1A* gene is monomorphic at all four loci of the *RsaI* enzyme in the given number of samples. Additionally, Sanger sequencing of a representative sample confirmed that the *MTNR1A* (856 bp) amplicon was monomorphic at all four *RsaI* cleavage sites.

However, Chu *et al.* (2007) have reported *RsaI* polymorphism at position G52A in seasonal estrous breeds (Liaoning Cashmere, Beijing native, Inner Mongolia Cashmere, and Wendeng milk goats) and year-round estrous breeds (Boer goats). The same G52A polymorphism has been reported in Honamli and Hair goats (Abdolahi *et al.,* 2019), and in Kacang and Peranakan Ottawa goats (Dagong *et al.,* 2019). However, this locus was found to be monomorphic in Jining Grey goats (Chu *et al.,* 2007), which is in agreement with our study on Beetal goats. It was noted that the

sample sizes in these studies, which reported G52A polymorphism, such as Chu *et al.* (2007), Agaoglu *et al.* (2015) and Dagong *et al.* (2019), were 150, 371, and 253, respectively; and the sample size of our study (n=35) is relatively small. Hence, 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 per cent identity and E-value ($<10^{-5}$).

Phylogenetic tree construction: The input 37 nucleotide sequences were used for phylogenetic tree construction. The T92+G was identified as the most suitable evolutionary model. The tree showed that the Beetal goat was positioned closely with the San Clemente goat and the ovine species, indicating high similarity and evolutionary conservation of the *MTNR1A* gene among them (Figure 2). The Beetal goat cluster was also located near other ruminants such as buffalo, cattle, and yak, reflecting their shared ancestry within the Bovidae family. In contrast, non-ruminant species and other vertebrates appeared on distant branches, suggesting greater evolutionary divergence of the *MTNR1A* gene in these species relative to the Beetal goat.

***Selection pressure analysis*:** The *MTNR1A* gene sequence within the species has primarily undergone neutral selection (p-value > 0.05), except in the San Clemente (p-value = 0.009).

***Secondary and tertiary structure prediction*:** Secondary structure prediction for the MTNR1A protein in Beetal goat revealed a combination of α-helix, strand, and coil structures, whereas the tertiary protein structure showed β-pleated sheets, α- helix, and loop structures (Figure 3). Ramachandran plot analysis revealed that more than 90% of the residues (225/241 and 236/241) fell within the favoured and allowed regions, respectively, indicating correct conformational prediction.

SUMMARY

The MTNR1A gene in female Beetal goats was found to be monomorphic for all four *RsaI* restriction sites. However, since the sample size is small, further validation with a larger population is recommended. The phylogenetic analysis indicated a high level of similarity and evolutionary conservation of the *MTNR1A* gene among goats and sheep. This gene has also primarily undergone neutral selection pressure between divergent species. The Ramachandran plot analysis confirmed the predicted three-dimensional structure of the MTNR1A protein in Beetal goat was conformationally correct.

ACKNOWLEDGMENT

The authors thankfully acknowledge the support rendered by the Department of Biotechnology, Government of India, for financial assistance through a scholarship to the first author during her M.Sc. Biotechnology and thank the Guru Angad Dev Veterinary and Animal Sciences University for providing the required resources and infrastructure.

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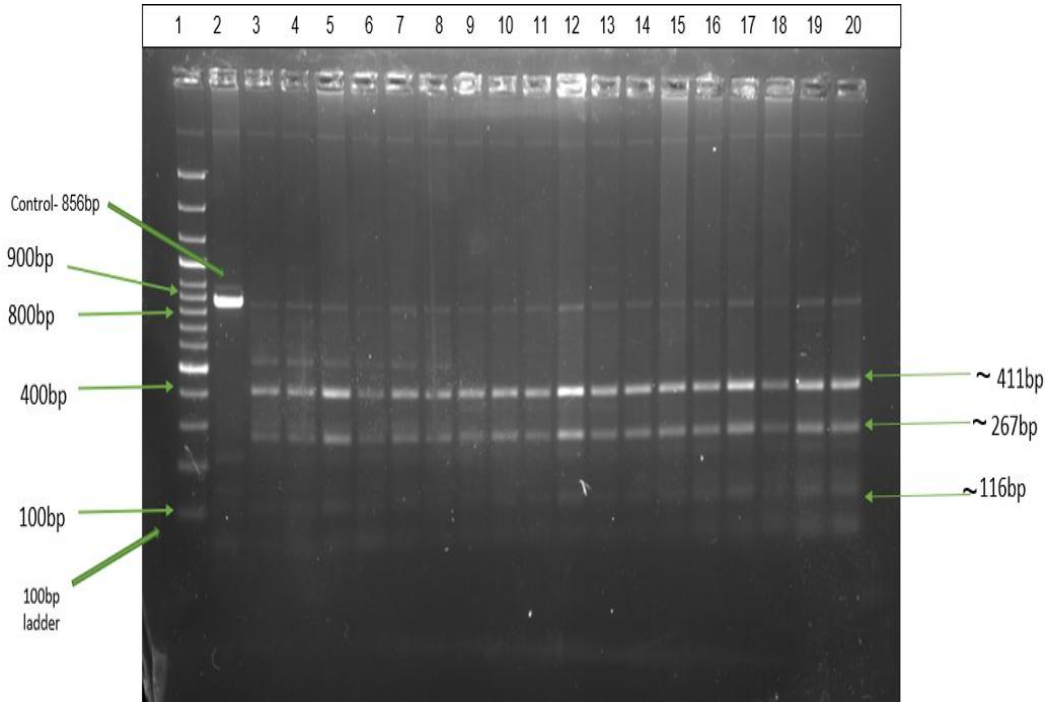

Figure 1: RFLP image of the *MTNR1A* gene by *RsaI* restriction enzyme on 2.0% agarose gel (lanes 3-20 are representative PCR products of the *MTNR1A* gene digested with the *RsaI enzyme,* and lane 2 consists of the control)

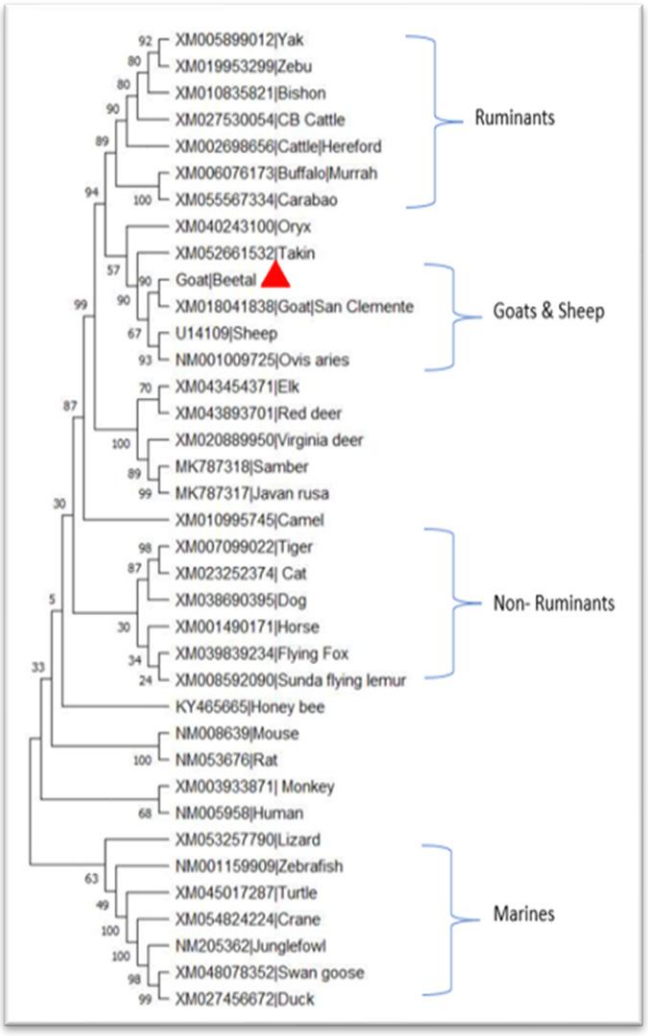

Figure 2: Phylogenetic tree of *MTNR1A* gene (*MTNR1A* gene of Beetal goat is denoted by a triangle

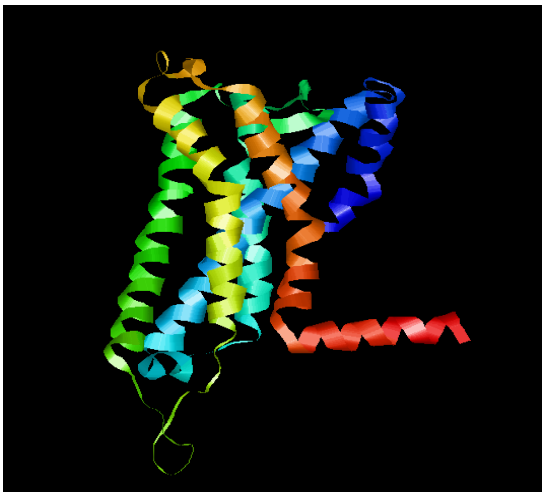

Figure 3: Predicted tertiary structure of MTNR1A protein in Beetal goat

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

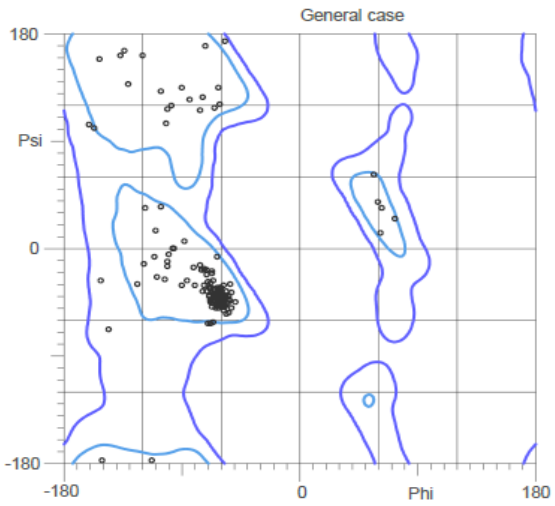

Figure 4: Ramachandran's plot analysis of amino acids present in the MTNR1A protein in Beetal goat using MolProbity

SUPPLEMENTARY MATERIALS

Table I: *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

Sequence Accession Number	Common Name	Scientific Name	Breed / Strain	Country	Sequence Type	Sequence Length
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

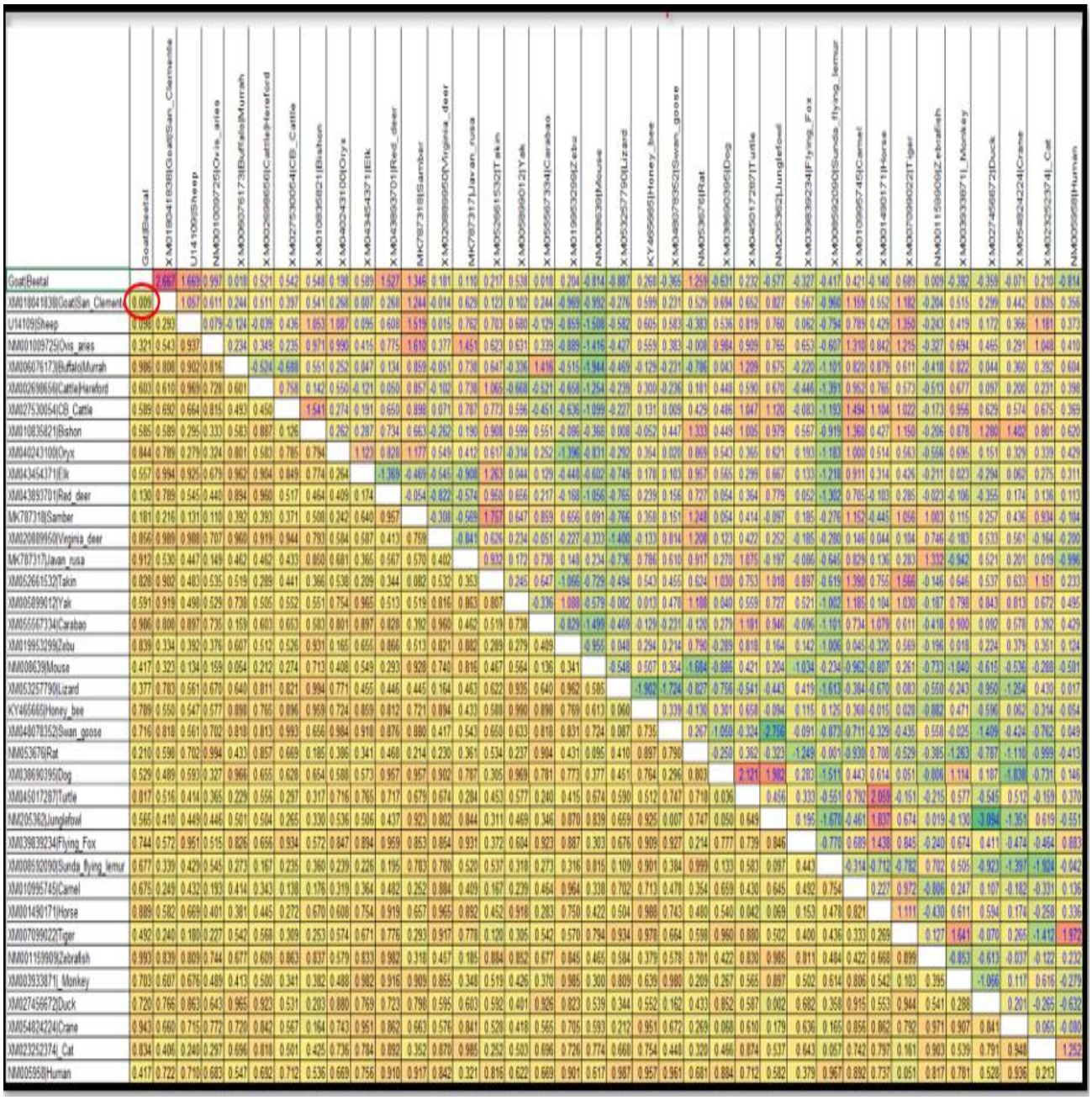

Figure 5: Neutral selection of the *MTNR1A* gene, estimated using MEGA 11 software

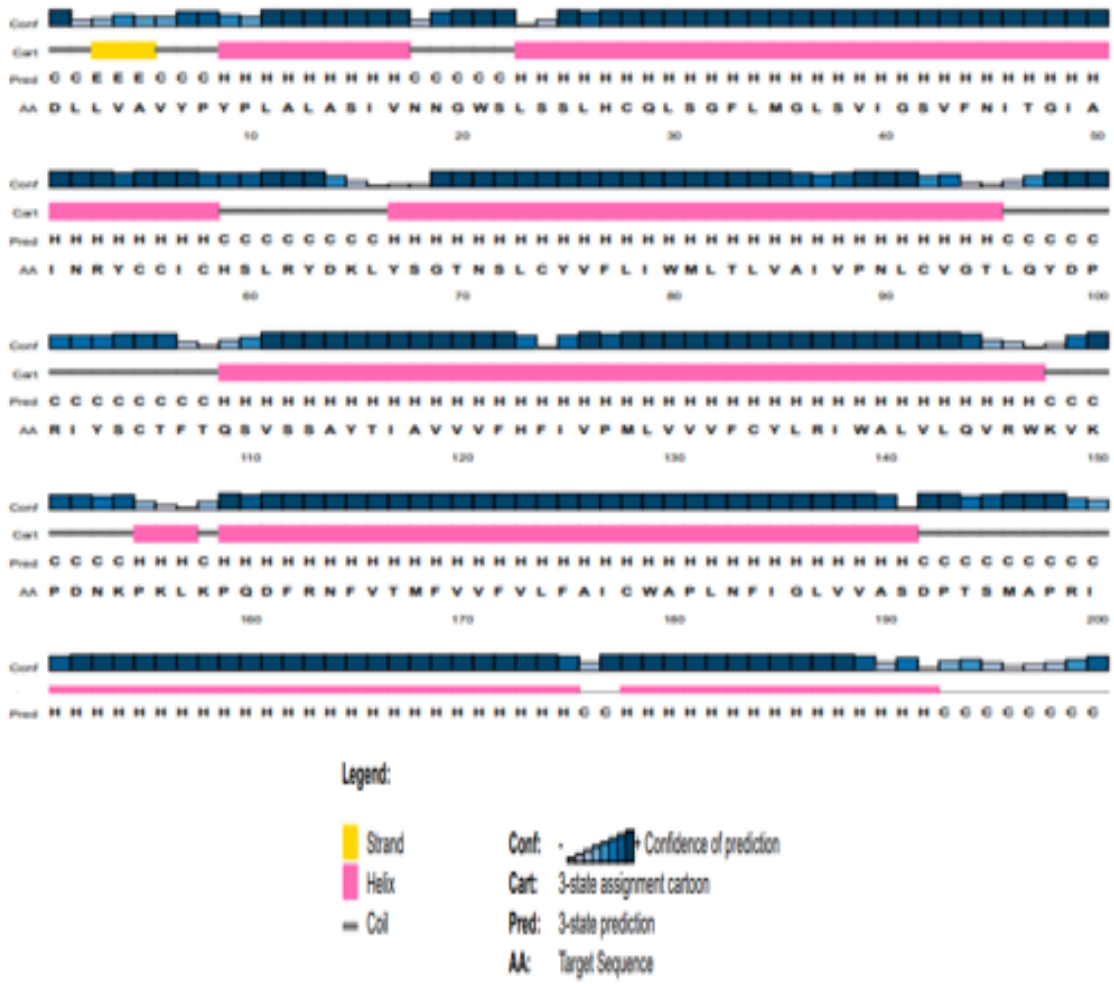

Figure 6: Secondary structure prediction of the MTNR1A protein in Beetal goat