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Mitochondrial ATPase 6/8 Genes Based Molecular Diversity and Phylogeny Analysis in Hog Deer (*Axis porcinus*)

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ABSTRACT

Hog deer (*Axis porcinus*) is a significant species in Pakistan due to its meat, skin and antlers, but there are insufficient molecular data, provoking us to explore its genetic variation and phylogenetic analysis using ATPase 6/8 genes of mitochondrial DNA. The blood samples from hog deer were collected and DNA was extracted by using in-house organic methods. PCR was used to amplify a total of 880 bp of overlapping genes, which were then sequenced. Bioedit software was used to align, and edit the sequences, and single nucleotide polymorphisms (SNPs) were evaluated. The MEGA X-based maximum likelihood method was employed to study the genetic relatedness between sequenced samples and reference sequence as well as other deer. Among query sequences, the greatest genetic distance was observed between #B2_N1 and #H2_N7 i.e., 0.1184 and smallest genetic distance is seen between #C2_N2 and #G2_N6 i.e., 0.0210. The genetic distance between query samples and 25 other deer nucleotide sequences was 0.37. The phylogenetic tree showed four nodes with bootstrap values less than 60 (56, 39, 39, 55) and were distantly related to other clades. The tree further showed that *Rucervus duvauceli (*swamp deer), *Axis axis* (chital deer), *Axis porcinus* and query sequences (B2_N1, H2_N7, C2_N2, G2_N6) form one clade being close relatedness. The study provided us with useful genetic analysis about *Axis porcinus* genetic variations and its phylogenetic relationships with related taxa, highlighting the importance to protect Pakistan's unique genetic resource.

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Authors' Contribution
MNA performed experiments. MM conceived the idea and supervised the study. TH helped in sample collection and manuscript writing.

Key words
***Axis porcinus*, Mitochondrial ATPase 6/8genes, Single Nucleotide Polymorphism, Molecular diversity, Phylogenetics**

INTRODUCTION

Hog deer (*Axis porcinus*) is considered as the ancient animal and Para hiran is the frequently used name of hog deers in Pakistan and India (Bhowmik and Chakraborty, 1999). Its conservation is important from an economic point of view to humans (Kanungo *et al*., 2012). Hog deer live in a highly complex environment. Their presence in the ecosystem is important as it is a prey species of the endangered Bengal tiger, *Panthera tigris*. It is threatened by habitat alteration, dispersal and illegal hunting (Gupta *et al*., 2018).

Mitochondrial DNA has many advantages over nuclear DNA. It is commonly confined in mitochondria (outside nuclear DNA) and is maternally inherited. Molecular markers of mitochondrial DNA have played an important role in the formation of links between closely related organisms (Hussain *et al*., 2015; Kumar *et al*., 2015). For studies of phylogenetics and recognition of various species, mitochondrial DNA markers have been used extensively in recent times (Abbas *et al*., 2017). The important features of mtDNA is its small molecular weight, basic structure, quick isolation, tissue generality and low recombination rate relative to nuclear markers (Hussain *et al*., 2015). The mtDNA has a very interesting characteristics such as very few spaces between genes, absence of introns, overlapping genes, and having genetic code (Garesse and Vallejo, 2001). It reveals hyper-diversity and is smaller than nuclear DNA of the whole genome and is suitable for population research (Vineesh *et al*., 2016).

The mitochondrial genome of the hog deer is 16,351 bp long. The nitrogenous base composition of Adenine, Thymine, Cytosine, Guanine is 33.4%, 29.3%, 24%, and 13.3%, respectively. The hog deer mt-genome contains 37 genes, out of which 13 are protein coding genes, two are rRNA genes and 22 are tRNA genes. The 13 protein coding genes are *ND1, ND2, COX1, COX2, ATPase8, ATPase6, COX3, ND3, ND4L, ND4, ND5, ND6, CYTB*. The two

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rRNA genes are 12S ribosomal RNA and 16S ribosomal RNA (Friedman and Nunnari, 2014; Hill *et al*., 2017).

The mitochondrial genes ATPase 6 and ATPase 8 are overlapping genes and are normally of different sizes in vertebrates (Luhariya *et al*., 2014). In hog deer, the size of both genes is 880 bp where ATPase 8 gene consists of 680 nucleotides and ATPase 6 gene consists of only 200 nucleotides. The start and stop codons of both the genes are the same, that is ATG, TAA respectively (Accession no MH443788).

Mitochondrial DNA genes ATPase 6/8 can be used to study the gap between taxa varieties and identify intraspecific variation in many species. Rapidly changing regions of the mitochondrial genome, such as ATPase 6/8 genes, are useful for high resolution study of vertebrate population structure (Kumar *et al*., 2017). In various fishes, ATPase 6/8 genes have been used to analyze both phylogeny and phylogeography. DNA-based recognition of the species of origin for beef and meat products are determined by ATPase 6/8 genes (Kumar *et al*., 2015). The genetic variations in ATPase 6/8 genes show the single nucleotide polymorphism (SNP). It helps to identify particular genetic characteristics like phylogenetic history, divergence, disease and evolutionary trends. It reveals the underlying mechanism of drug efficacy and tracking ancestral migration. It helps researchers mapping genomes for disease and traits as it remains evolutionary stable (Razali, 2014). By using phylogeny based studies, one can evaluate the extinction rates of hog deer (Nee *et al*., 1994).

The present study investigates the genetic variation in terms of SNPs and these are used to construct a phylogenetic tree using mitochondrial ATPase 6/8 genes of hog deer.

MATERIALS AND METHODS

The blood samples of *Axis porcinus* (hog deer, n= 5) were collected and brought to the lab from the Lahore Zoo and Safari Park, Lahore. The samples were transported in an icebox and stored at -20 °C till use. DNA was extracted from blood samples by following the phenol chloroform-isoamyl alcohol method with little modification. The extracted DNA was quantified through NanoDrop One/ One^{C} and visualized under BIO-RAD Gel DOC™ XR+. The samples with highest concentration were selected for PCR optimization.

Polymerase chain reaction (PCR)

PCR was used for amplification of selected mitochondrial ATPase 6/8 genes. 25 µl of reaction mixture was prepared with 25 ng of DNA. DNA and primer dilutions were briefly vortexed and short spun, and 0.2 ml PCR tubes were used. Touchdown PCR with the difference of 0.5°C backward from 55°C to 51°C (55-54.5-54-53.5-53-52.5-52-51.5-51 °C) for nine cycles of extension was used. 4µl of PCR amplified sample was mixed with 2 µl loading dye (bromophenol blue) and then loaded on to the gel. In the first well, 2 µl of gene ruler™ 1 kb DNA ladder was loaded for measuring other samples. Amplicon samples were run on 2% gel at 90 volts for 45 min. The primers for amplification of mitochondrial ATPase 6/8 genes (Accession No. MH443788) were made using Primer3 software V 4.0.0. The forward and reverse primers were:

F: 5′-GCCTTTTAAGCTAGAGACTGAGAGC-3′
R: 5′-TGTTAGGGGTCAGGGACTTG-3′

Sequencing of PCR product

For sequencing the PCR product, five samples of PCR product along with forward primer of ATPase 6/8 genes was sent to Lab Genetix, Lahore. The product was purified by using Exo SAP IT™ PCR product cleanup and then cycle sequencing was done by using BigDye Terminator V3.1 kit according to the relevant temperature on verity thermal cycler. The subsequent sample was sequenced by SeqStudio Genetic Analyzer by Applied Biosystems.

Data analysis by bioinformatics tools

Sequencing results were analyzed by using BioEdit. NCBI BLAST was used to ascertain the nature of nucleotides and to check the resemblance with reported sequences. The evolutionary history was inferred using the neighbor-joining method. ATPase 6/8 genes of mitochondrial DNA from hog deer were compared to other deer species and related animals in a phylogenetic analysis. Mega X software was used for assembling, aligning and editing the sequence.

RESULTS

Four samples (B2_N1, C2_N2, G2_N6, H2_N7) of ATPase 6/8 genes were sequenced, blasted using nucleotide BLAST and each sequence was matched to the mitochondrial genome of *Axis porcinus* having accession number MH443790.1. The query coverage of three amplified sequences show maximum similarity B2_N1=99%, C2_N2=100%, G2_N6=100% whereas H2_N7 show significant diversity (94%). Table I shows the query coverage of four sequences in NCBI: The selected sequences at the 5′ and 3′ ends of the four samples were trimmed to have most appropriate sequences.

Table I. Trimming of nucleotides of ATPase 6/8 genes against the Sequence ID.

Sequence ID	Original sequence length	Nucleotide removed at 5′ end	Nucleotide removed at 3′ end	Remaining sequence length
B2_N1	873	64	23	786
C2_N2	927	64	251	612
G2_N6	965	74	362	529
H2_N7	895	54	45	796

Sequence analysis among these hog deer within the sequenced data was done by using DnaSP.

SNP was found in sequences of *Axis porcinus* using reference sequence with accession number MH443790.1. This analysis showed that out of 786 nucleotides in B2_N1, 64 nucleotides indicated SNP. Similarly, out of 612 nucleotides in C2_N2, 23 nucleotides showed polymorphisms. In 529 nucleotides of G2_N6, 15 bases showed SNP while in 796 nucleotides long H2_N7, 89 nucleotides showed polymorphism. The total number of haplotypes (h) was 4 and haplotype diversity (Hd) was found to be 1.0000, with the variance of haplotype diversity being 0.03125.

Table II shows the genetic distance among query sequences of *Axis porcinus.* There are 821 positions and the total genetic distance among the query sequences was 0.0500. The distances were determined by applying the p-distance method and the unit of the genetic distance is number of base differences per site.

Table II. Genetic distances among query sequences of *Axis porcinus.*

Genetic sequences	#B2_N1	#C2_N2	#G2_N6	#H2_N7
#B2_N1				
#C2_N2	0.0483			
#G2_N6	0.0344	0.0210		
#H2_N7	0.1184	0.0512	0.0267	--

Greatest genetic distance is observed between #B2_N1 and #H2_N7 i.e., 0.1184. Smallest genetic distance is observed between #C2_N2 and #G2_N6 i.e., 0.0210.

Genetic distances among query samples and 25 other nucleotide sequences and an outgroup (*Alces alces*) were studied and interpreted by MEGA X. Gaps between nucleotide sequences were removed and the whole data contained 514 positions. The whole genetic distance among all the sequences was 0.37. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was 1000 replicates (Fig. 1).

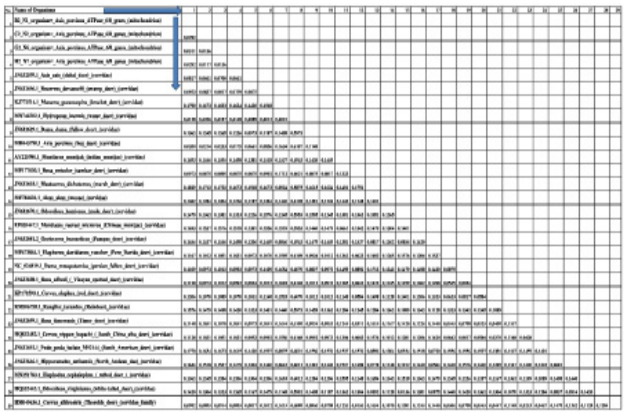

Fig. 1. The genetic distance among various species of deer with query sequences of ATPase 6/8 genes of *Axis porcinus*. The unit genetic distance is the number of base differences per site. Greatest genetic distance is observed between C2_N2 and water deer (*Hydropotes inermis*) that is 0.621 and smallest genetic distance (0.012) is observed between C2_N2 and H2_N7.

Bootstrap values are present at each node and this is the basis of the phylogenetic tree. Bootstrap values higher than 60 are classified as genetically similar. These values indicate that how close species are to each other genetically. In this phylogenetic tree, four nodes showed the bootstrap value less than 60 (56, 39, 39, 55) which indicated that they were not closely related with other clades in this tree. The phylogenetic tree showed that our sequences are more closely related to *Axis axis* (chital deer) (Fig. 2).

The query sequence, B2_N1 is closely related with H2_N7 sequence than C2_N2 and G2_N6. C2_N2 sequence and G2_N6 sequence both have a common ancestor. Furthermore, *Rucervus duvauceli (*swamp deer), *Axis axis*, *Axis porcinus* and query sequences (B2_N1, H2_N7, C2_N2, and G2_N6) form one clade. It means that *Recervus duvauceli* is more closely related to *Axis axis* as its bootstrap value is 95 and further *Axis axis* is very close to *Axis porcinus*. The query sequences have 100 bootstrap values with our reference sequence, *Axis porcinus*. Our sequences (B2_N1, H2_N7, C2_N2, G2_N6) form one clade (Fig. 3).

DISCUSSION

Hog deer is considered to be the least studied species in Pakistan and it is declared endangered in this region (Abbas *et al*., 2017). ATPase 6 and ATPase 8 genes provide molecular diversity and phylogenetic analysis of *Axis porcinus*. Single nucleotide polymorphisms (SNPs)

were explored by comparing the nucleotides of sequenced genes with reference sequence (MH443790.1). This genetic variation shows the phylogenetic relationship of query sequence with other deer species.

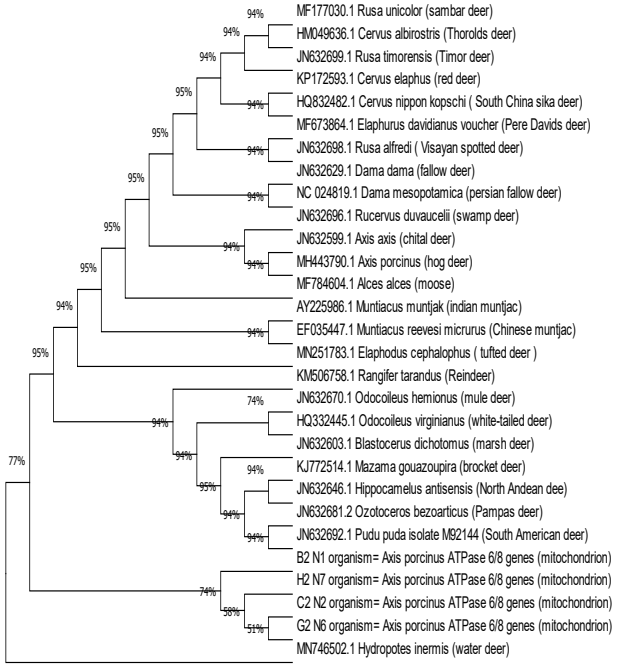

Fig. 2. Phylogenetic tree with bootstrap values of query sequences with other deer species.

The sequencing results showed that H2_N7 has more diverse types of polymorphism compared to the other three sequences. The BLAST results proved that ATPase 6/8 sequences of query samples are similar to *Axis porcinus* present around the world which means that there is unexpressive difference in the sequences of mitochondrial sequences ATPase 6/8 genes. The genetic distances reflect the difference among query sequences (B2_N1, H2_N7, C2_N2, and G2_N6) to judge the degree of their relatedness. Greatest genetic distance means how far apart two sequences are, and is seen between B2_N1 and H2_N7 that is 0.1184, while the smallest genetic distance is seen between C2_N2 and G2_N6 that is 0.0210.

Genetic distance between query samples (B2_N1, H2_N7, C2_N2, and G2_N6) and other deer families shows the relatedness of the samples. Greatest genetic distance is observed between C2_N2 and water deer (*Hydropotes inermis*) that is 0.621 which means that *Axis porcinus* is very distinct morphologically from water deer whereas the smallest genetic distance (0.012) is observed between C2_N2 and H2_N7 indicating a close evolutionary relationship.

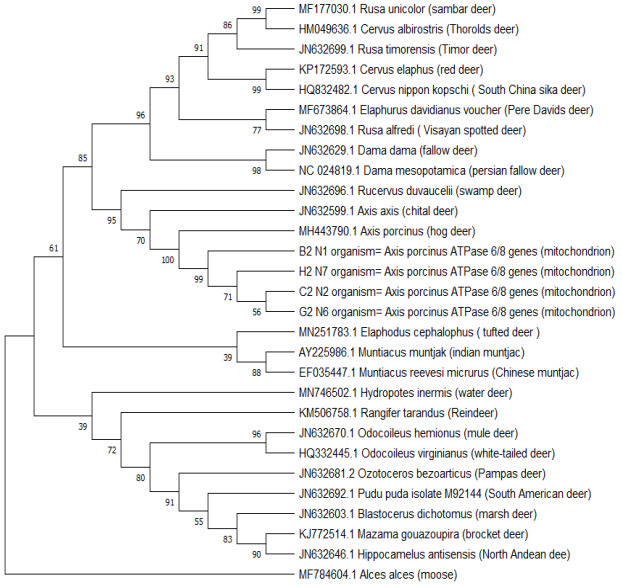

Fig. 3. Phylogenetic tree of deer species with respect to query sequences using ATPase 6/8 genes. The reference sequence shows 100 bootstrap value with our query sequence. Moreover, it appears from this phylogenetic tree that *Axis porcinus* is closely related to cheetal deer (*Axis axis*).

It was inferred from the phylogenetic tree that query sequences of ATPase 6/8 genes matched the reference sequence of *Axis porcinus* (MH443790.1) with a bootstrap value of 100. It was noted that *Axis axis* was more closely related to *Axis porcinus* through maximum likelihood analysis of phylogenetic tree. These findings are also supported by Hill *et al*. (2019) that the hog deer is closely related to *Axis axis* through mitochondrial genes (Cyt b, COI custom-made D-loop) and nuclear genes (α-lactalbumin gene and protein kinase C iota). Furthermore, swamp deer, Persian fallow deer, fallow deer, Visayan spotted deer, Pere David's deer, South China sika deer, red deer, Timor deer, Thorold's deer and sambar deer are more closely related with our sequences (B2_N1, H2_N7, C2_N2, and G2_N6).

SNPs in four query samples were analyzed by using reference sequence (MH443790.1). It was observed that at position 333, all the four query sequences showed a change of nucleotide (adenine) from the reference sequence (guanine). Furthermore, 22 mutations were found in *Axis porcinus* and 13 mutations were seen in *Ovis vignei punjabiensis*. Hence, *Axis porcinus* belongs to a different family consisting of a large number of species in Pakistan (Hussain *et al*., 2015).

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Statement of conflict of interest

The authors have declared no conflict of interest.

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