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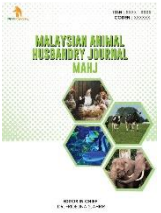

RESEARCH ARTICLE

THARPARKER INDIAN PEAFOWL (*Pavo Cristatus)* GENETIC ARCHITECTURE; EXPLORED BY MITOCHONDRIAL DNA D-LOOP MARKER

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ABSTRACT

The blue peafowl from the genus Pavo is the largest species among both of its wild and domestic types, which is widely distributed in the habitats of Pakistan, India, Nepal, Sri Lanka and Bangladesh. The aim of this study is to have an insight of molecular diversity and phylogenetic analysis of *Pavo cristatus* on the basis of mitochondrial D-loop region. A total of six samples were collected from the Lahore Zoo and Safari Park Lahore. The whole genome was extracted by using standard protocol with minor modification. PCR amplification was done by using a set of mitochondrial D loop primer. Codon Code Aligner 5.1.5 was used for the sequence alignment and data analysis. Three C/T heterozygous loci were found at position 168, 170 and 223. One A/G heterozygous locus was observed at position 234. One insertion of A at 137, one transversion C>G at 190 and three transition mutation were observed at position 206, 222 and 236 respectively. Phylogenetic analysis was performed with the help of MEGA 6 software using neighbor joining method which revealed that our individuals are closely related with the Japanese *Pavo cristatus*. Moreover, this Japanese and Pakistan species sharing their common ancestor. Our samples are clearly placed themselves in one clade while all other related species are in another clade which predict the substantial divergence between Pakistani and other studies D loop regions of peacocks.

KEYWORDS

Blue peafowl, *Pavo cristatus*, DNA D loop, Phylogenetics, Pakistan

1. INTRODUCTION

The Tharparker Indian Peafowl, scientifically known as *Pavo cristatus*, is a magnificent bird species that belongs to the Phasianidae family. This species is native to the Indian subcontinent and has also been introduced to various other countries (Nameer et al., 2020). The Tharparker Indian Peafowl is renowned for its stunning appearance, with the males, known as peacocks, displaying vibrant blue and green plumage along with an extravagant train of feathers (Heffner et al., 2020). The females, called peahens, possess a more subdued appearance, showcasing a white face and iridescent green lower neck. This genus contains three species belonging to different region with different appearances. These three spices are *Pavo cristatus* (blue peafowl), *Pavo muticus* (Green Peafowl) and *Afropavo congensis* (Congo Peafowl) (Morishita et al., 2022).

First described by Carl Linnaeus in 1758, the Tharparker Indian Peafowl was given the scientific name *Pavo cristatus*. The term "cristatus" refers to the crested appearance of the peafowl (Gustafsson et al., 2023). The Tharparker Indian Peafowl is primarily found in the Indian subcontinent, inhabiting the drier lowland areas of Sri Lanka. It thrives in a variety of habitats, including moist and dry-deciduous forests, cultivated regions, and areas around human habitations. The blue peafowl is also known as Indian peafowl or common peafowl which is the native breed of India, Pakistan, Sri Lanka and Nepal but rare in Bhutan and nearly extinct in Bangladesh (Adesh et al., 2021). This specie population has also been introduced in other counties like USA, Hawaii Islands, Europe, Australia, South Africa and New Zealand. It is the national bird in India and Provincial bird of Punjab in Pakistan (Rajpar, 2022).

There are many color mutants of blue peafowl in Pakistan and India such as: black shoulder peafowl, white peafowl, and pied etc. *Pavo muticus* (green peafowl) spread in Burma, Indochina, Thailand, Malaysia, and Java (Intarapat et al., 2023). But currently it is only available in Java that's why these are sometimes also called as Java green peafowl or Burmese. It is bigger in size then the common blue peafowl and it is also listed as vulnerable by IUCN (Kushwaha and Kumar, 2016). Congo peafowl known as the mvule, is a species of peafowl native to the Congo Basin. It differs noticeably from the above discussed peafowl in its appearance and size also. It also has a featherless neck. It is a very rare, little-known species and is found in the tropical forests of Zaire (Africa) (Dong et al., 2021).

Peafowl are variety of beautiful colored mutants that's why they are reared in many areas of sub-continent. They exist in a broad range of habitats which includes tropical and subtropical or in evergreen and deciduous forests, grassland and farmland areas. They take their forage from ground and build their nests on the ground but rest on the top of trees (Talha et al., 2018). It is reported that according to International Union for Conservation of Nature has listed Indian peafowl as Least Concern species because their population is widespread and semi feral in above mentioned

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Asian countries. The relative relationships of blue peafowl to the peacock pheasant, other pheasants, and Congo peafowl are still somewhat unclear, but it looks like that *Afropavo* (Congo peafowl) is the closest living relative of blue peafowl proved their relationship on the basis of karyotype similarities (Rajpoot et al., 2021).

The Tharparker Indian Peafowl is listed as a species of Least Concern on the IUCN Red List. Despite this, conservation efforts are essential to protect their natural habitats and prevent any potential threats to their population (Kumaraand Yogendra, 2023). The peafowl's cultural significance, celebrated in Hindu and Greek mythology, has played a role in conserving this majestic species. In recent years, genetic studies have shed light on the Tharparker Indian Peafowl's genetic diversity and population structure. One such study focused on analyzing the DNA D loop, a non-coding region of the mitochondrial genome. The DNA D loop contains valuable information about the species' evolutionary history and genetic relationships (Naseer et al., 2018).

Advanced molecular techniques were utilized in the study to get DNA samples from individuals belonging to several groups of Tharparker Indian Peafowls. After then, these samples were sequenced and examined to find patterns and genetic variants (Liu et al., 2022). In order to better understand genetic variety within species, researchers have attempted to assess the genetic diversity and phylogenetic analysis of the blue Indian peafowl specie in this article. These analyses are based on the D-loop area of the mitochondria, which has been characterized and studied. In an organelle, mitochondrial DNA is found outside the nucleus (Yacoub Haitham and Badwy Moataz, 2019). The DNA of mitochondria has a non-coding section known as the Control region, which contains a displacement loop, also known as a D-loop.

Because mitochondrial DNA is constantly present in excess, it is exploited. According to some experts, the rate of evolution of mitochondrial DNA is five to ten times faster than that of nuclear DNA (Kumar Mariappan et al., 2023). Additionally, it is passed down through the maternal line directly, bypassing recombination or crossing over. As a result, all DNA molecules are interpreted as a single genetic unit with several alleles. They can also be readily observed because they are relatively smaller in size than nuclear DNA. Because the D-loop regulatory region of mtDNA experiences more mutations per sequence than other mtDNA regions, this region is being used by the researchers. For this reason, studying all of its allele types in the D-loop sequence is simpler.

2. Materials And Methods

2.1 DNA Extraction

Blood was used to extract genomic DNA using the Qiagen technique (QIAamp DNA Mini Kit, Cat No./ID: 51304). 350 µl of Lysis Buffer (1 M Tris, 0.5 M EDTA, 5 M NaCl, 10% SDS) was mixed with 1 milliliter of blood samples. The mixture was centrifuged for 5 minutes at 13,000 rpm, and the pellet was taken out. This process was repeated at least three times. Next, 40–50 µl of proteinase K and 70 µl of 10% SDS were added. The mixture was centrifuged, and the supernatant was taken out. An equal volume of phenol: chloroform: isoamyl alcohol (5:24:1) was added, which was then incubated for 10–15 minutes, centrifuged at 13,000 rpm for 3–5 minutes, and the pellet was taken out.

After adding 0.5 ml of isopropanol to the samples and centrifuging once more, the pellets were cleaned with 70% ethanol and centrifuged once more for ten minutes. Samples were left out to dry for a full day. Samples of DNA were dissolved in either 200 µl of TE buffer or water treated with DEPC. After that, a nanodrop technique was used to measure the quantity and purity of DNA samples at a wavelength of 260/280 nm (Sefc et al., 2003).

2.2 PCR amplification

The Primer 3 program was used to create primers for PCR amplification (Untergasser et al. 2012). GbCR4.L (5'-CGA TTC ATG GTA GCA GGT CA-3') and CSB1.H (5'-AAC ATG TCC AAC AAG CAT TCA-3') were the primers we employed (Mullis et al., 1986). The isolated DNA was then amplified in a total volume of 25 µl using a second PCR reaction (Mullis et al., 1986). Initial denaturation at 96 °C for 5 minutes is followed by 30-35 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds, followed by a final extension at 72 °C for 10 minutes in PCR amplification.

2.3 Sequencing and phylogenetic analysis

After cleaning and 70% ethanol washing, PCR samples were sent for sequencing using a Beckman Coulter kit, and the outcomes were examined. Additional software was utilized for data processing, such as BEAST v1.10.4 (Tamura et al., 2013) for phylogenetic analysis and Codon Code Aligner Version 5.1.4 (Codon Code Corporation) for sequence alignment and analysis. Additionally, Bio Edit v7.0.9.1 was used to process the sequences. The DNaSP v4.1 program was used to estimate haplotype and nucleotide diversity (Librado and Rosaz 2009). From NETWOK 4.5.1.2, we selected the tree with the highest likelihood value as our best using the maximum parsimony (MP) technique (Bandelt 1999).

The coalescent-based model used in BEAST v1.10.4 was then used to reconstruct the species trees. For every analysis, the two mtDNA loci were divided according to codons (Tamura et al., 2013). Using the first high variation section fragment (LVH), a tree made of 481 base pairs was created. A phylogenetic analysis was performed using the reference sequence, which was a prior sequence obtained from GenBank, to determine the haplogroup present in the current isolate. Historical population statistics are included in genetic variation data. Demographic events are typically investigated using mtDNA of mismatch distribution (for comparison) (Slatkin and Hudson 1991; Rogers and Harpending, 1992).

Two standards have been developed: expanding populations have a uniform single peak distribution, while multimodal distribution principles do not require a constant population size. JModeltest 2.1.7 identified the suitable DNA model analysis prior to the system phylogeny study. Using the proper Bayesian information criteria, the optimal model of HKY+1 for neighbor connection parameters was identified in this investigation. PhyML 3.1 was utilized by BioNJ for the analysis of the first tree. Non-parametric pseudo-random is supported by estimating the maximum probability node by a 100-start analysis. MrBayes 3.2 was used to do Bayesian analysis (Geyer, 1991). Lastly, the study yielded 10.000.000 generations for all species sequence data (100% of bootstrap support) using a random starting shirt.

Every 100 generations, it has been examined and is widely used to verify generation and transition using the Markov Chain model. NETWOK 4.5.1.2 is used to perform the pairwise difference matrix (Bandelt et al., 2001). The non-parametric bootstrap, also known as just the bootstrap from here on, is a computer-based statistical method that estimates values of interest by resampling data. A phylogenetic tree is then inferred by analyzing the bootstrap sample. In phylogenetic analysis, the precise link between bootstrap values, Pboot, and posterior probability values, $P(\tau \mid D)$, is a crucial and unanswered subject. It has been suggested that even while each measure's theory is essentially independent, they ought to be comparable. Further research on the behavior and connection between the bootstrap and posterior probability measures is therefore necessary. Unfortunately, for this investigation, an analytical solution is not immediately evident. The bootstrap values for 1.000 simulated samples for a single point in the model space were compared using a paired design in this experiment.

3. Results And Discussion

While captive Tharparker Indian Peafowl manage to tick the box for minimal genetic variation, the true need for allelic diversity paints a far starker picture. With a mere 27% of the required breeding pairs present, the captive program sputters along, desperately short of fuel. Political and logistical barricades further choke the vital flow of genes, potentially pushing these dazzling birds perilously close to a genetic abyss. The Tharparker Indian Peafowl's solitary struggle against extinction throughout the late 20th century serves as a stark reminder of our capacity to overlook looming threats. While seemingly adaptable to food supply changes, populations faced a silent onslaught of unidentified dangers, leading to their precipitous decline (BirdLife International, 2007). The widespread implementation of "peafowl parks" without thorough scientific backing exemplifies the perils of superficial solutions (Shwe et al., 2021). True conservation demands a shift towards research-driven, genetically robust captive breeding programs to ensure the future of these magnificent birds.

However, amidst the desolation, flickers of hope ignite. The valiant fight to ban diclofenac in Pakistan bears fruit, offering a potential reprieve for the beleaguered peafowl. Captive breeding programs, though challenged by genetic bottlenecks, strive to maintain their diversity and pave the way for future reintroductions. Conservation efforts take root, aiming to mend and stitch together the fragmented tapestry of their natural habitats (Gu et al., 2022; Hussein et al., 2022). The future of the Tharparker Indian Peafowl hangs in the balance. But by shedding light on the dark corners of their plight, implementing robust conservation strategies, and unlocking the secrets of their resilience, we can rewrite their story. Only then will these feathered jewels reclaim their rightful place in the vibrant tapestry of

Pakistan's ecosystems, their plumage gleaming not just with iridescent beauty, but with the light of a hard-won survival.

Seven samples were sequenced in order to find single nucleotide polymorphisms (SNPs), but one sample produced no findings. Five heterozygous situations were found for the four SNPs (Table 1). When compared to the reference sequence (C/T), C>T and A>G conversions were found at positions 168, 170, 223, 236, and 234, respectively (Table 1). Even though mtDNA is haploid, heteroplasmy the presence of multiple mtDNA types in a single animal can happen. The occurrence of heterozygous places in our sequences can be explained by heteroplasmy, as it is currently believed that all persons are heteroplasmic to some extent, much over the limits of detection in DNA sequence analysis. Table 1 contains all other genetic information.

Table 1: SNPs Heterozygous conditions and mutation types

Position	RefSeq A.C: D66900.1	Changed position	Total number of samples	Transition/Transversion
137	-	A	6	Insertion
168	T	C/T	6	
170	C	C/T	6	
190	G	C	V-3, V-6	Transversion
206	T	C	6	Transition
222	T	C	6	Transition
223	C	C/T	6	
234	A	A/G	6	
236	C	C/T	6	Transition

Changes in breeding success directly impact genetic diversity, the cornerstone of a species' future resilience. A population with dwindling offspring carries a smaller gene pool, increasing susceptibility to disease, environmental changes, and other unpredictable events. By pinpointing colonies experiencing severe declines in breeding, conservation efforts can prioritize interventions to bolster reproductive rates and safeguard the Tharparker Indian Peafowl's precious genetic tapestry.

Looking at the results of molecular diversity and a phylogenetic analysis of Tharparker Indian Peafowl (*Pavo cristatus*), using a mitochondrial D-loop marker, new cluster was identified. Phylogenetic tree was constructed by using MEGA 6. Sample seven is originated from same clad as reference sequence. Their common ancestor also related with samples five. While sample 3, 4, 6 and 1 share common ancestor with sample 5, 7 and reference sequence. Following is the phylogenetic tree of samples with other species. All the samples have common ancestor of reference sequence (*Pavo cristatus* A.C: D66900.1). The threes species of genus *Pavo* (*P. cris, P. mut, and A. congensis*)share common ancestor with a different species *Meleagrisgallopavo.*

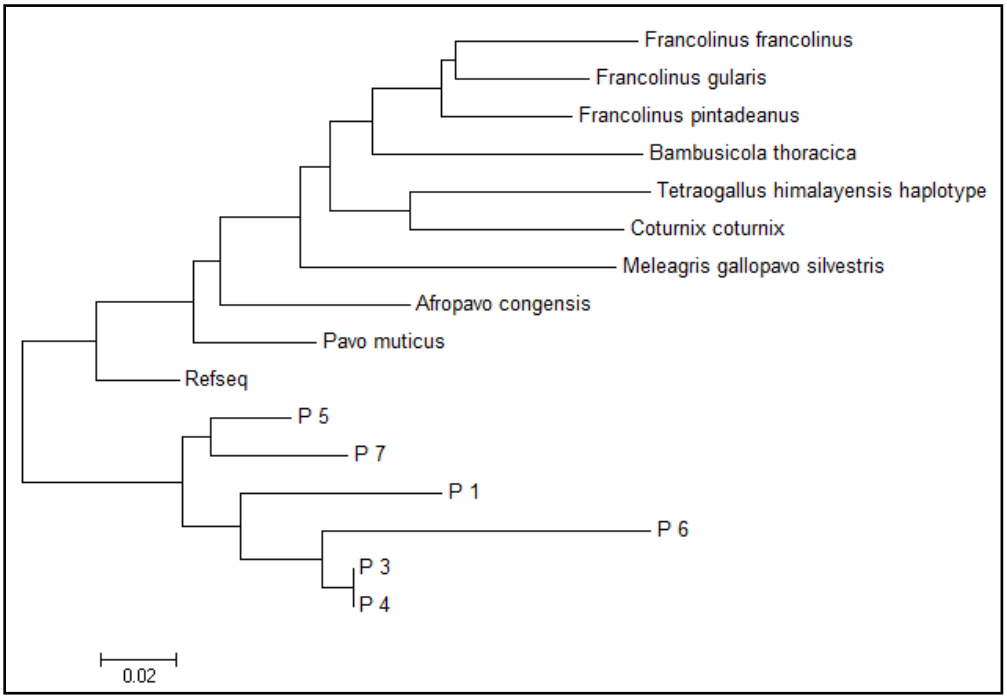

Figure 1: Phylogenetic Tree of *Pavo cristatus* samples and other Species

The genetic study revealed fascinating insights into the Tharparker Indian Peafowl's genetic makeup. Researchers discovered a high level of genetic diversity within the populations, indicating a healthy and robust species. The analysis of the DNA D loop allowed for the identification of distinct haplotypes, providing evidence of evolutionary divergence and gene flow between populations. Interestingly, the genetic study also uncovered potential genetic markers associated with specific traits or adaptations. These findings open up opportunities for further research into the genetic basis of the Tharparker Indian Peafowl's unique characteristics.

Understanding the genetic diversity and population structure of the Tharparker Indian Peafowl is crucial for effective conservation efforts.

This genetic study provides valuable information for conservationists and policymakers to develop targeted strategies for the species' preservation. By identifying genetically distinct populations and understanding their relationships, conservationists can prioritize conservation efforts in areas of high genetic importance. While the genetic study on the Tharparker Indian Peafowl's DNA D loop has provided significant insights, there are still many avenues for future research. Expanding the scope of genetic analysis to other regions and populations could reveal additional patterns and variations. Furthermore, investigating the genetic basis of specific traits, such as the development of the elaborate train, could deepen our understanding of the peafowl's evolutionary history.

4. Conclusion

The Tharparker Indian Peafowl, with its stunning plumage and captivating courtship displays, continues to fascinate researchers and nature enthusiasts alike. Genetic studies, such as the analysis of the DNA D loop, offer valuable insights into the species' genetic diversity and evolution. With the knowledge gained from these studies, we can better conserve and protect this iconic bird for future generations to appreciate and admire.

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