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RESEARCH ARTICLE

THE CASE OF PEAFOWL AND PORCUPINE KILLING: MOLECULAR PERCEPTION OF A HEINOUS WILDLIFE CRIME

[1]Thakare, V. J., [2,*]Pande, A. A. and [3]Thakare, P. V.

[1,2]Regional Forensic Science Laboratory, Behind Police, Commissioner's Office, Chandur Railway Road, Amravati: 444606, Maharashtra, India
[3]Biotechnology Department, Sant Gadge Baba Amravati University, Amravati, Maharashtra, India

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ABSTRACT

We address a recent case where the forest officials found suspected flesh of nearly 10 peafowls and quills, bones with tissue remains of porcupine. Apart from quills, flesh and tissue bone mixture could not be differentiated on the basis of physical paramaters. Cytochrome oxidase I gene of the mitochondrial DNA was used for the identification of species. Sequence analysis revealed that the DNA obtained from flesh was of Indian peafowl which is included under Schedule I of The Indian Wildlife Protection Act, 1972 and DNA obtained from bones with tissue was of Indian crested porcupine which is also protected under The Indian Wildlife Protection Act, 1972 amended upto 2002.

INTRODUCTION

India's national bird since 1963, the peacock (*Pavo cristatus*) is protected under schedule I of The Indian Wildlife Protection Act, 1972. The killing of a peacock is strictly prohibited and as per section 51(1-A) attracts imprisonment which may extend to seven years and also a fine which shall not be less than ten thousand rupees. Despite the law, there are umpteen deaths and reduction in number of the species is seen at places where they were in abundance earlier. Poaching of peacock is done as their oil is considered as an aphrodisiac and for use as an ingredient in many Siddha preparations. As the Indian crested porcupine (*Hystrix indica*) adapts to a wide range of habitats and food types, it is listed by the IUCN as Least Concern as of 2008. It is protected under schedule IV of the Indian Wildlife Protection Act, 1972 amended up to 2002. They are widely hunted as they are destructive to gardens and agricultural crops. A large trade of these species exist for consumption and medicinal use. Conservationists have estimated that porcupines are reducing by 10% every year now. If they go, they will leave the environment much poorer, because they are extremely important ecologically in spreading seeds and pollen. Definite identification of species is necessary for conviction under various wildlife protection acts. The only available evidence is pieces of meat, skin or bones in cases of suspected poaching. In such cases species identification can be done using molecular techniques. Analysis of trace evidence samples can be achieved by application of DNA based techniques to the investigation of wildlife crime. Mitochondrial DNA (mtDNA) testing has become a standard procedure in species identification as there is no recombination of mtDNA. All maternal descendants will have the same mitochondrial DNA sequence with the exception of mutations and all loci will be linked (Clayton, 1982; Hayashi et al., 1985).

As compared to only two copies of nuclear DNA there are multiple copies of mitochondrial DNA (Robin and Wong, 1988). For forensic species identification, genetic loci are derived from taxonomic and phylogenetic studies (Simon et al., 2006). In the present case, cytochrome oxidase I (Linacre and Tobe, 2009; Rastogi et al., 2007) was used as marker. It is adopted by Barcode for Life Consortium http://www.bol dsystems.org (Hebert et al., 2003; Borisenko et al., 2008).

Brief history of the case

Forest officials found suspected flesh and bones of nearly 10 peafowls near a farm and bones and tissue with quills of porcupine in the river bed near the farm. Samples were collected and sent to Regional Forensic Science Laboratory, Amravati for species identification.

***Corresponding author:* Pande, A. A.**
Regional Forensic Science Laboratory, Behind Police, Commissioner's Office, Chandur Railway Road, Amravati: 444606, Maharashtra, India.

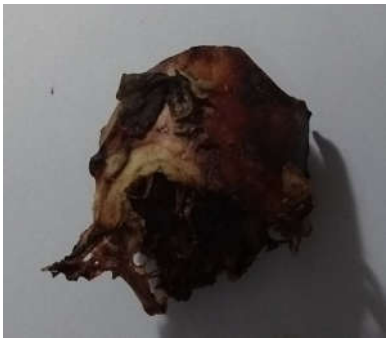

Fig. 1.

Fig. 2.

DNA extraction from the samples

DNA from flesh sample, bone sample was extracted using manual extraction protocol. (Figure 3). DNA was isolated from the tissue attached to the bone of exhibit 1 and 2 and tissue attached to the quill. Agarose gel electrophoresis was used for separation of genomic DNA and the DNA bands were visualized on gel documentation system. (Alpha Innotech, USA)

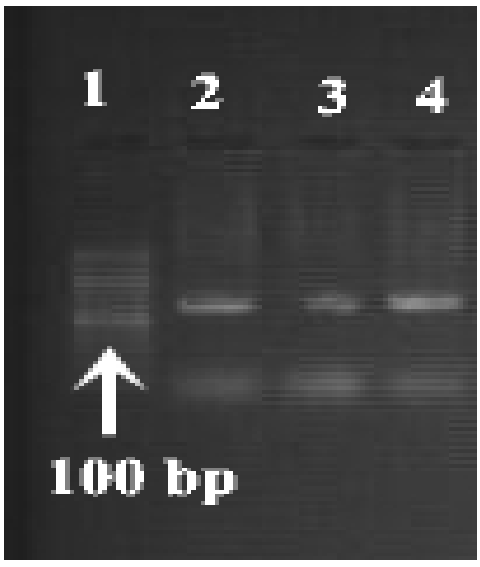

Fig. 3. Gel image showing bands of total isolated DNA

- Lane 1: 100 bp ladder
- Lane 4 : Bone with tissue and quills
- Lane 2: Bone with tissue
- Lane 5 : Bone with tissue and quills
- Lane 3:Bone with tissue

DNA amplification using Polymerase Chain Reaction (PCR)

The amplification of *coi* (cytochrome oxidase I) gene of mitochondrial region was used for PCR amplification using universal primer pair that consistently amplified around 700-bp fragment of *coi* across the broadest array of animal orders. Primer pairs used for DNA amplification targeted single copy mitochondrial DNA. Thermal cycling was performed in 0.2 ml thin walled PCR tubes with 20 µl reaction volume. PCR products were analyzed by electrophoresis on 1.2% agarose gels and visualized by staining with ethidium and amplified DNA band of approx. 700bp. Amplified DNA i.e., *coi* gene. Thermal cycling was performed in 0.2 ml thin walled PCR tubes with 20 µl reaction volume. PCR products were analyzed by electrophoresis on 1.2% agarose gels and visualized using ethidium bromide (Fig. 4).

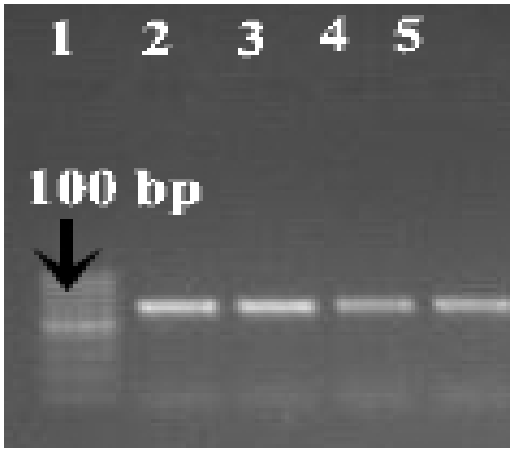

Fig. 4. DNA amplification of coi locus of mitochondria.

- Lane 1: 100 bp ladder
- Lane 4 : Bone with tissue and quills
- Lane 2: Bone with tissue
- Lane 5 : Bone with tissue and quills
- Lane 3:Bone with tissue

DNA Sequencing

Amplified DNA i.e., of *coi* gene obtained, were sequenced in ABI 3500 genetic analyzer using ABI Big Dye TM Terminator Cycle sequencing kit by Chromous Biotech Pvt. Ltd, Bengaluru, Karnataka, India. The sequence of DNA from tissue and bones from exhibit no. 1 and 2 are shown in fig 5 and 6 respectively, while the nucleotide sequences of meat sample attached to quill are shown in 7 and 8 respectively. All the samples were aligned and after comparison in clustalW and analysed in CLC bench work, it was noted that two samples were of one animal and the rest of two samples were from other animal. The sequences obtained sample 1 and 2 were searched using BLAST and the sequences showed match with *Pavo cristatus* i.e. Indian Peacock. Fig 10 and 11 shows the BLAST alignment showing match of 97% with *Pavo cristatus* species. A dendogram was constructed using UPGMA with boostrap of 1000 replications the nucleotide sequences of the sample 1, 2 and the sequences downloaded from the Blast search of the sequences. The dendogram is shown in fig 13. The sample were analyzed for *coi* nucleotide sequences. It was found that the nucleotide sequence for *coi* were same and belonged to same animal.

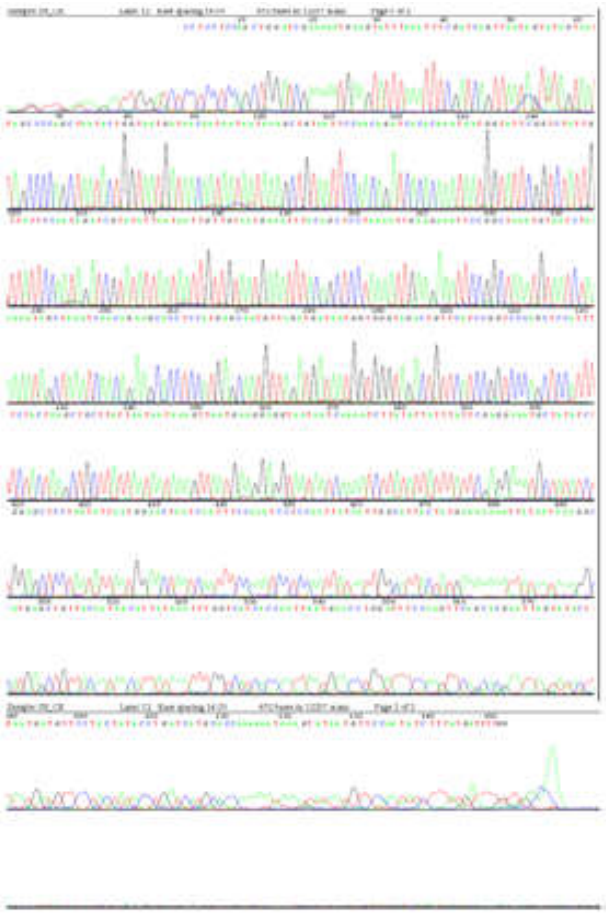

Fig. 5. Nucleotide sequence chromatogram of bone sample exhibit no 1.

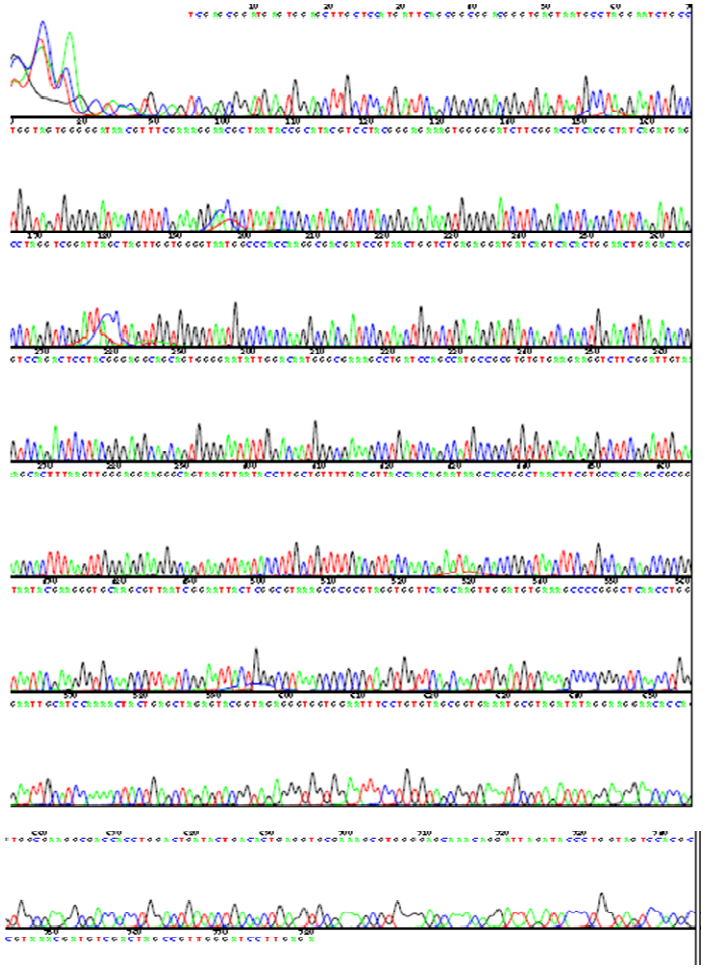

Fig. 6. Nucleotide sequence chromatogram of bone and tissue sample exhibit no 2.

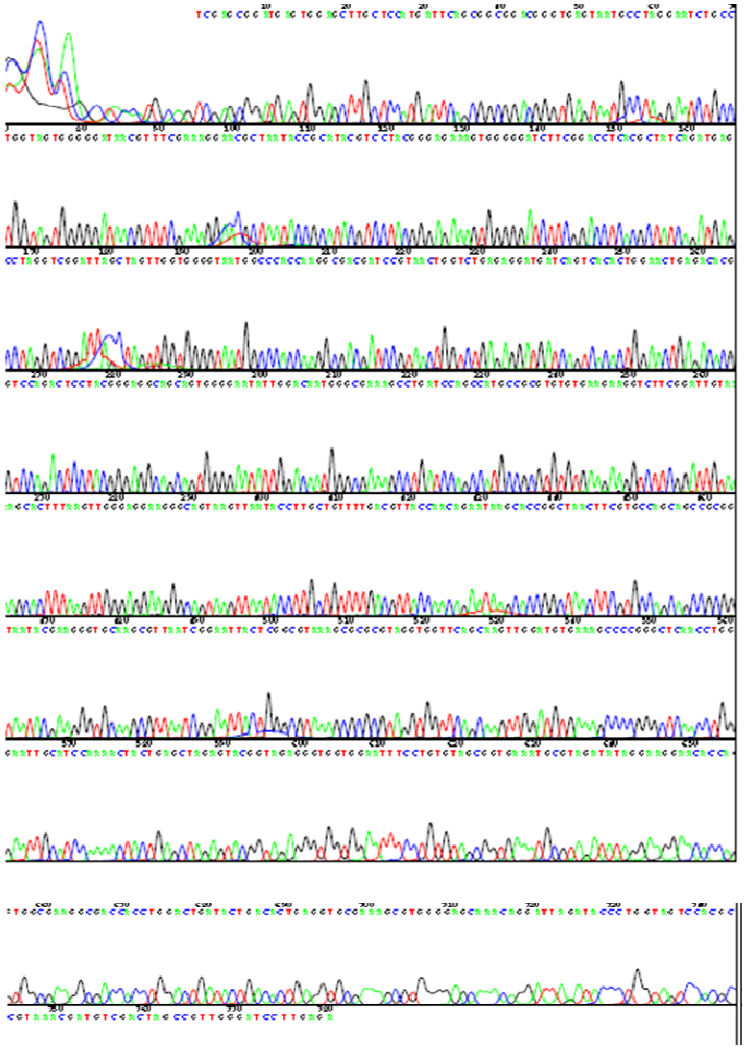

Fig. 7. Nucleotide sequence chromatogram of meat attached to quill.

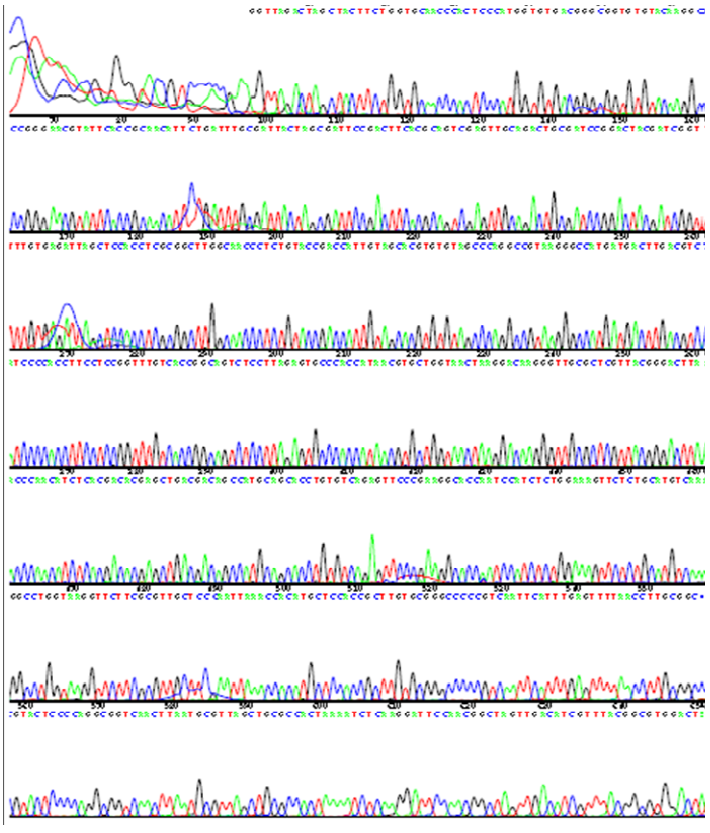

Fig. 8. Nucleotide sequence chromatogram of tissue from quill.

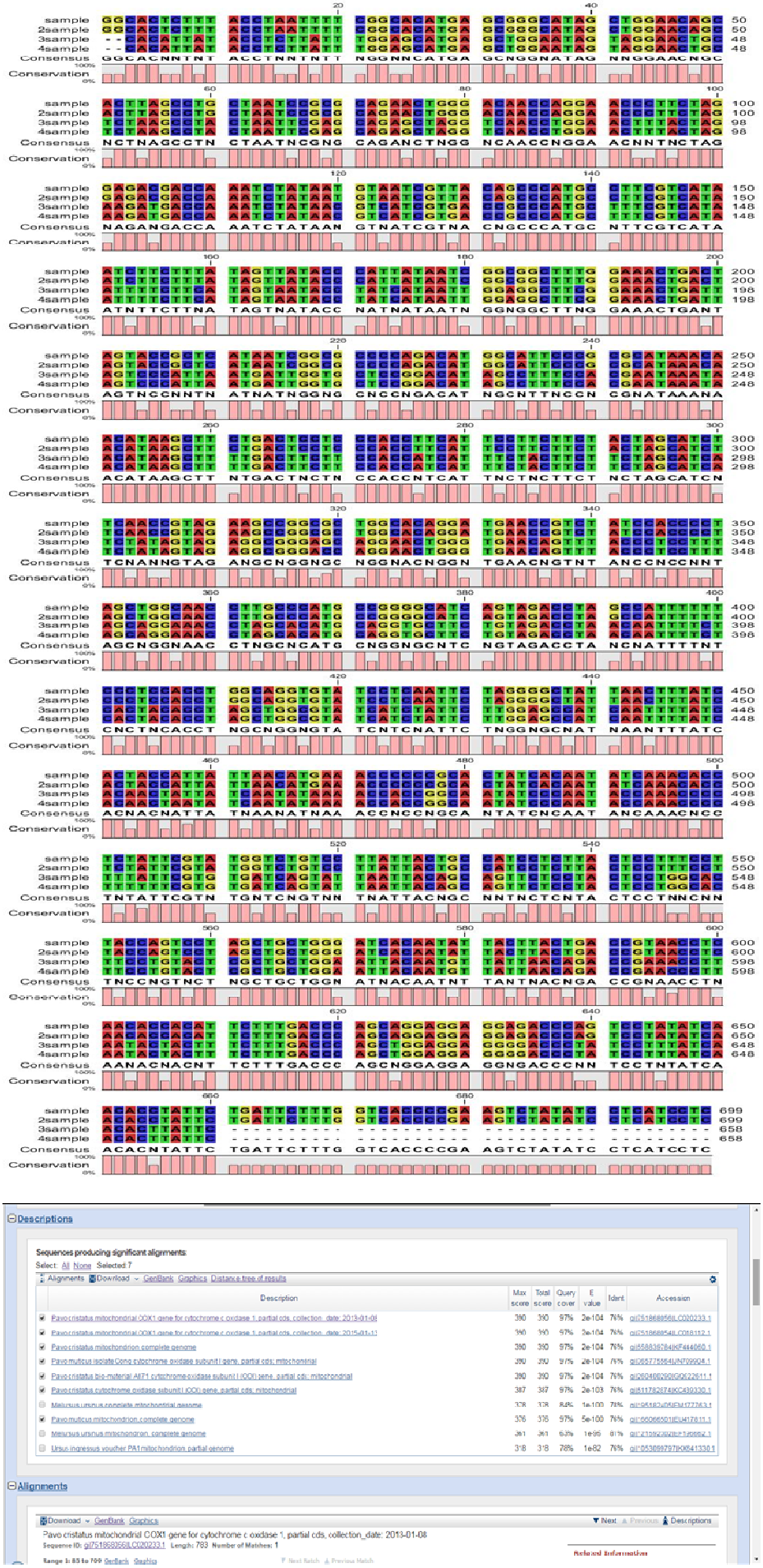

Fig. 10. Blast alignment for the nucleotide samples

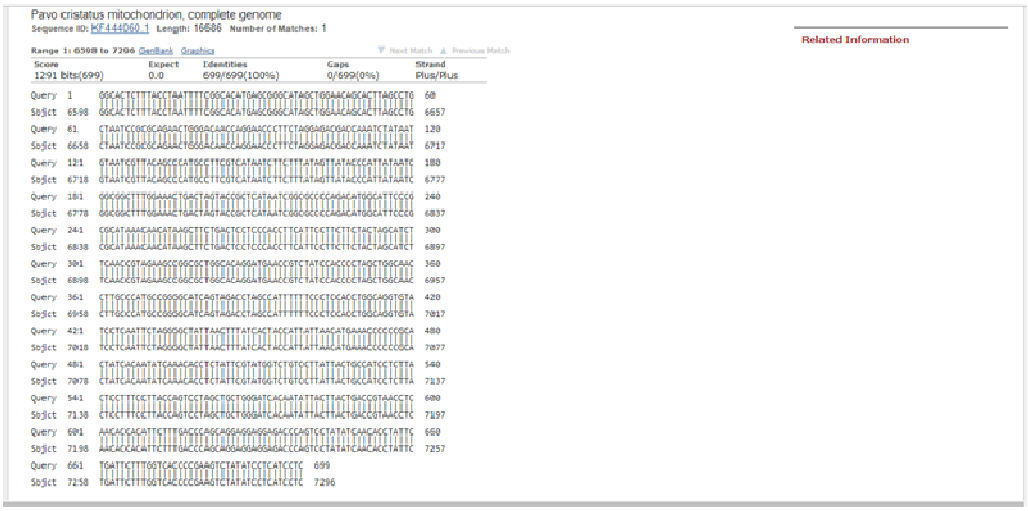

Fig. 11.BLAST search of nucleotide sample 1.

Fig. 13. Dendogram of nucleotide sequences of samples 1, 2 and Blast related sequences.

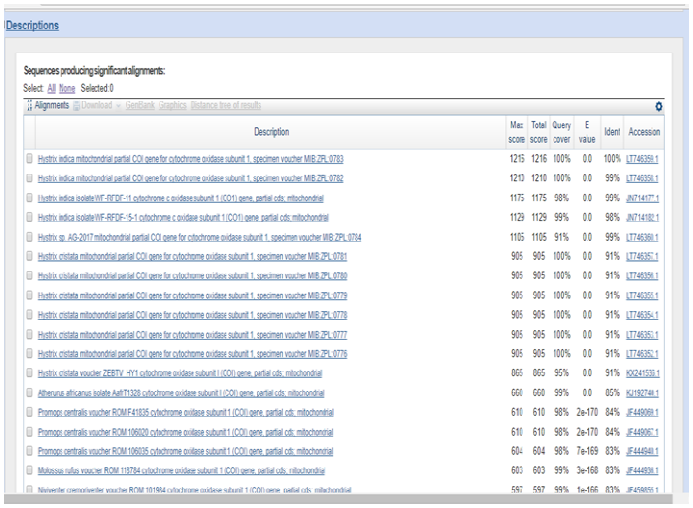

Fig. 14. Blast alignment for the nucleotide sample 3.

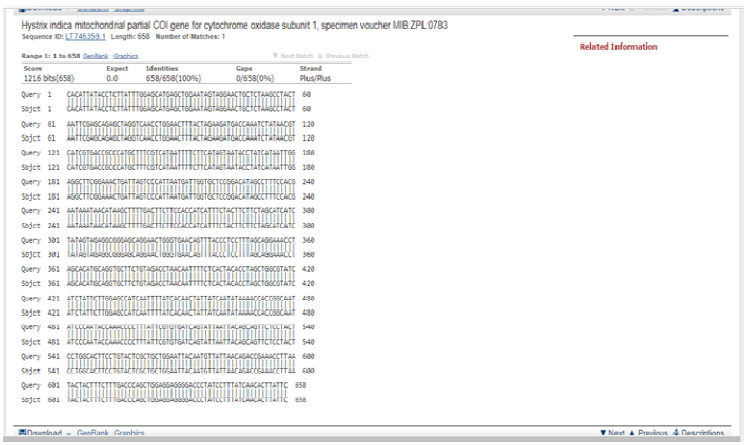

Fig. 15. BLAST search of nucleotide sample 3

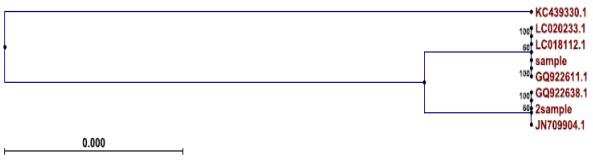

Fig. 16. Dendogram of nucleotide sequences of samples 3, 4, Blast related sequences and Atherurus africanus.

The sequences also showed a match with related species *Pavo muticus,* upto 96%, however the literature cited showed *Pavo muticus* (green peacock) is a species of peacock that is found only in North east India ranging to Bhutan. The sequence match was 100% *Pavo cristatus.* The dendogram revealed that *Pavo muticus* (EU417811) was out grouped and thus was not the sample provided for analysis. Upon detail analysis of the sample, it was found to be of *Pavo cristatus* (also called as Indian Peacock or मोर in marathi) based on DNA barcode analysis as well as the nucleotide sequence analyzed from NCBI (National Centre for Biotechnological Information, USA). The contig match with *Pavo cristatus* up to 100% and species demonstrated that the samples were of *Pavo cristatus.* The sample or exhibit no 3 and 4 were from different animal. Fig 2 indicated that the animal quill phenotypically matched with that of *Hystrix indica* with alternate brown black and white bands. The tissue attached at the bottom and tissue samples were processed for isolation of DNA, PCR and amplification of *coi* gene. The sequences of the exhibit 3 and 4 were found to be same. (Fig. 9) The nucleotide sequences were subjected for nBlast search and results showed that sample belongs to *Hystrix indica* (Fig. 14 and 15). A dendogram was constructed using UPGMA with boostrap of 1000 replications the nucleotide sequences of the sample 3,4 and the sequences downloaded from the Blast search of the sequences including *Atherurus africanus*. The dendogram is shown in fig 16.

The sample 3 and 4 were analyzed for *coi* nucleotide sequences. It was found that the nucleotide sequence for *coi* were same and belonged to same animal. The sequences also showed a match with related species *Hystrix cristata,* upto 100% and also with *Hystrix indica*. The literature cited showed that *H cristata* i.e. Indian crested porcupine was referred as *H. indica* by some authors. Thus being the same species nucleotide sequences were submitted with different name but is one species. The sequence match was 100% *H.indica or H. cristata.* The dendogram revealed that *Atherurus africanus* (KC439330.1) was out grouped as it was the African Brush-tailed Porcupine. Upon detail analysis of the sample, it was found to be of *H.indica* (also called as Indian crested procupine or सायाळ in marathi) based on DNA barcode analysis as well as the nucleotide sequence analyzed from NCBI (National Centre for Biotechnological Information, USA).

DISCUSSION AND CONCLUSION

Peacocks eat grain, berries, flowers, seeds, seedlings, tender shoots, as well as insects, lizards, frogs and snakes. Although peacocks are protected under the wildlife laws and export of their tail feathers and articles made by them continues to be banned by India and also under CITES (Convention on International Trade in Endangered Species), the gathering and selling (within the country) of claimed to be naturally shed peacock feathers, is not illegal. A single peacock normally sheds or moults 150-200 feathers annually. As the demand for peacock plumes grows, naturally shed long tail-eyed feathers are simply not enough and peacocks are increasingly killed. The Indian crested porcupine is protected under the India Schedule IV of the Indian Wildlife Protection Act of 1972, amended upto 2002. Because they are destructive to agricultural crops and gardens, porcupines are widely hunted. Moreover, as a result of urbanization, infrastructure development and pesticide use, suitable porcupine habitat is currently declining. A large trade of these porcupines exists for consumption and medicinal use. All the Indian species which are classified under CITES appendix I and Wildlife Protection Act, 1972 schedule I, outlaws international commercial trade of these species and also catching or killing of such species in India amounts to imprisonment up tp seven years and fine not less than ten thousand rupees (http://www.cites.or g/eng/app/reserve.php; http://www.envfor.nic.in/division/wildlife). In this case, identification was done with the help of COI gene. This method is reliable as it shows versatility in its ability to use a single conserved primer pair to accurately identify, if conspecific sequences were available in database. Careful sampling and analysis thereof helped in precise identification of the samples which lead to expose a wildlife crime.

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