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The complete mitochondrial genome of *Gyps coprotheres* (Aves, Accipitridae, Accipitriformes): A correlation between phylogeny and susceptibility to diclofenac toxicity among raptors

Emmanuel Adawaren [Corresp., 1], **Morne Du Plessis** [2], **Essa Suleman** [3], **Duodane Kindler** [3], **Almero Oosthuizen** [2], **Lillian Mukandiwa** [4], **Vinny Naidoo** [5]

[1] Department of Paraclinical Science/ Faculty of Veterinary Science, University of Pretoria, Pretoria, Gauteng, South Africa

[2] Bioinformatics and Comparative Genomics, South African National Biodiversity Institute, Pretoria, Gauteng, South Africa

[3] Molecular Diagnostics, Council for Scientific and Industrial Research, Pretoria, Gauteng, South Africa

[4] Efficacy and Safety, Australian Pesticides and Veterinary Medicines Authority, New South Wales, Australia

[5] Paraclinical Science/ Faculty of Veterinary Science, University of Pretoria, Pretoria, Gauteng, South Africa

Corresponding Author: Emmanuel Adawaren
Email address: adawarenvet1@yahoo.com

Three species of Old World vultures on the Asian peninsula are slowly recovering from the lethal consequences of diclofenac. At present the reason for species sensitivity to diclofenac is unknown. Further it has since been demonstrated that other Old World vultures like the Cape (*Gyps coprotheres*) (CGV) and griffon (*G. fulvus*) vultures are also susceptible to diclofenac toxicity. Oddly the New World Turkey vulture (*Cathartes aura*) and Pied crow (*Corvus albus*) are not susceptible to diclofenac toxicity. As a result of the latter, we speculate an evolutionary link to toxicity. As a first step in understanding the susceptibility to diclofenac toxicity, we use the CGV as a model species for phylogenetic evaluations, by comparing the relatedness of various raptor species known to be susceptible, non-susceptible and suspected by their relationship to the Cape vulture mitogenome. This was achieved by next generation sequencing and assembly. The Cape vulture mitogenome has a genome size of 16908 bp comprising of 13PCGs, 22tRNA, 2rRNA and a control region called the D-loop. Phylogenetic analysis showed a relationship between mitogenome phylogeny and diclofenac susceptibility among Old World vultures, Spilornis, Nisaetus, Aquila eagle species and other suspected members of the Accipitridae family. Susceptibility to diclofenac toxicity thus appears to be due to evolutionary reasons.
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The Complete Mitochondrial Genome of Gyps coprotheres (Aves, Accipitridae, Accipitriformes): A correlation between phylogeny and susceptibility to diclofenac toxicity among raptors

Adawaren Emmanuel[1], Du Plessis Morne[2], Suleman Essa[3], Kindler Duodane[3], Oosthuizen Almero[2], Mukandiwa Lilian[4], Naidoo Vinny[1]

[1]Department of Paraclinical Science, Faculty of Veterinary Science, University of Pretoria, Gauteng, SOUTH AFRICA

[2]South African National Biodiversity Institute, Department of Bioinformatics and Comparative Genomics, Pretoria, Gauteng, SOUTH AFRICA

[3]Council for Scientific and Industrial Research (CSIR), Department of Molecular Diagnostics, Pretoria, Gauteng SOUTH AFRICA

[4]Australian Pesticides and Veterinary Medicines Authority, Department of Efficacy and Safety, New South Wales, AUSTRALIA

Corresponding author: Emmanuel Adawaren, adawarenvet1@yahoo.com; +27747749213

Faculty of Veterinary Science, Department of Paraclinical Science, University of Pretoria, Pretoria, South Africa

1.1 Abstract

Three species of Old World vultures on the Asian peninsula are slowly recovering from the lethal consequences of diclofenac. At present the reason for species sensitivity to diclofenac is unknown. Further it has since been demonstrated that other Old World vultures like the Cape (*Gyps coprotheres*) (CGV) and griffon (*G. fulvus*) vultures are also susceptible to diclofenac toxicity. Oddly the New World Turkey vulture (*Cathartes aura*) and Pied crow (*Corvus albus*) are not susceptible to diclofenac toxicity. As a result of the latter, we speculate an evolutionary link to toxicity. As a first step in understanding the susceptibility to diclofenac toxicity, we use the CGV as a model species for phylogenetic evaluations, by comparing the relatedness of various raptor species known to be susceptible, non-susceptible and suspected by their relationship to the Cape vulture mitogenome. This was achieved by next generation sequencing and assembly. The Cape vulture mitogenome has a genome size of 16908 bp comprising of 13PCGs, 22tRNA, 2rRNA and a control region called the D-loop. Phylogenetic analysis showed a relationship between mitogenome phylogeny and diclofenac susceptibility among Old World vultures, Spilornis, Nisaetus, Aquila eagle species and other suspected members of the Accipitridae family. Susceptibility to diclofenac toxicity thus appears to be due to evolutionary reasons.

Keywords: Cape vulture, Accipitridae, Phylogeny, Susceptibility, Diclofenac toxicity, Cytochrome C oxidase

1.2 INTRODUCTION

Generally, vultures may be classified as Old or New World vultures based on the apparent convergent evolutionary scavenging feeding habit (Seibold, Helbig, 1995). However, in reality, they are morphologically and evolutionary diverse group of birds (Seibold, Helbig, 1995, Wink, 1995, Johnson *et al*., 2016) . Old world vultures are descendants of the Accipitridae family comprising of eagles, hawks, kites and buzzards (Wink, 1995; Lerner and Mindell, 2005; Clements *et al.,* 2019). According to Clements taxonomic classification, Accipitridae is one of the largest non-passerine members of bird species comprising of 252 species (227 are monophyletic while 25 are polyphyletic) belonging to the order Accipitriformes (Clements *et al*., 2019). Furthermore, families of bird species included in the Accipitriformes order are Accipitridae, Sagittariidae, Pandionidae and Cathartidae with the latter now classified under the order Cathartiformes (Clements *et al*., 2019).

Raptors are predator birds that hunt and kill their prey and they also include those who feed on carrion. Bird species known as raptors belong to the Accipitridae, Falconidae, Cathartidae, Strigidae and Tytonidae families (Clements *et al*., 2019). Raptors are valuable indicators of habitat quality based on their ecological sensitivity as predators and scavengers (Lerner, Mindell, 2005). However, vultures belonging to the Accipitriformes order are currently facing devastating drops in their population numbers from an array of problems ranging from loss of their natural habitat, collision with high-tension electric cables and wind turbines, intentional poisoning of animal carcasses by poachers of endangered wildlife species and accidental ingestion of pharmaceutical contaminated animal carcasses (Ogada *et al*., 2016, Naidoo *et al*., 2017, Adawaren *et al*., 2018). One notable incident was the near complete extinction of three Gyps vulture species (White-rumped Vulture- *Gyps bengalenesis,* Indian Vulture- *G. indicus*, and Slender-billed Vulture- *G. tenuirostris*) in India, Nepal and Pakistan from the consumption of carcasses from animals dosed with diclofenac before dying (Oaks *et al*., 2004; Swan *et al*., 2006b; Naidoo *et al*., 2009a; Adawaren *et al*., 2018). At present it is estimated that the drug caused the deaths of over 10 million vultures in the region (Naidoo *et al*., 2017). Furthermore, diclofenac has been incriminated to cause the death of a steppe eagle (*Aquila nipalensis*), a member of the Accipitridae family with classical sign of toxicity seen in vultures (Sharma *et al*., 2014). This incidence raises concern on the vulnerability of the Accipitriformes and other raptors to diclofenac toxicity. The Cape vulture has also been reported

to be susceptible to the toxic consequences of diclofenac (Swan *et al*., 2006b, Naidoo *et al*., 2009b, Naidoo *et al*., 2010, Naidoo *et al*., 2017, Adawaren *et al*., 2018)

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID) mostly understood for its analgesic, anti-inflammatory and an antipyretic characteristic is used for the treatment of inflammatory disorder in human and animals. However in vultures the drug, present in the meat of carcasses the vulture fed upon, was sufficient to cause death within 48 hours of exposure with signs of renal failure associated with visceral and articular gout being evident on necropsy (Oaks *et al*., 2004, Swan *et al*., 2006b, Naidoo, Swan, 2009). While the general mechanism of action of the NSAIDs and their toxicity in mammals is well characterized, the same cannot be said for vultures. Despite the exact cellular mechanism underlying toxicity remaining unknown, the clinical progression of toxicity is well established (Swan *et al.*, 2006b, Naidoo *et al*., 2009a). Vultures generally show signs of depression as early as 24 hours after exposure (depression charaterized by head drooping, reluctance to move, open wings, decreased appetite, loss of aggression). At approximately 48 hours post exposure, the affected animals usually succumb with characteristic gross post-mortem lesions of visceral and articular gout and histological lesions of renal tubular necrosis, especially the proximal convoluted tubules (PCT) of the kidney and hepatocytes of the liver (Swan *et al*., 2006b, Naidoo *et al*., 2009a). At the physiological level, the drug is associated with massive elevation in plasma uric acid amount, acidaemia, hyperkalaemia and increases in plasma liver enzyme activity. In terms of the temporal relationship, the first signs of depression correspond to the first elevation in uric acid amount indicative of early kidney damage, followed by increase in plasma liver enzyme activities indicative of hepatic necrosis, and lastly death associated with hyperkalaemia and acidosis. While speculative, the increase in potassium is purported to induce cardiac failure and death (Naidoo *et al*., 2007).

Following the discovery of diclofenac as the cause for the vulture deaths, research has shown that these Old World bird species are also vulnerable to other NSAIDs toxicity with the notable exception of meloxicam thus far (Swan *et al*., 2006a, Naidoo *et al*., 2010, Adawaren *et al*., 2018). The scenario is, however, different with the New World vultures. In a study in which the Turkey vulture (*Cathartes aura*) was exposed to concentration of diclofenac (25 mg/kg) 100 times above the median lethal dose (0.1-0.2 mg/kg) in Old World vultures, no overt toxicity was

evident. Furthermore, the diclofenac could hardly be detected in the tissue after necropsy, with the concurrent pharmacokinetics study demonstrating a low plasma half-life of elimination of 6 hour, in comparison to 12-16 hours observed in Cape vultures (Rattner *et al*., 2008, Naidoo *et al*., 2009a). The high sensitivity of the Old World vulture also contrasts with other bird species whereby high doses in the region of 10 mg/kg was needed to induce toxicity in chickens (*Gallus gallus*) with a corresponding plasma half-life elimination predicted within the range of 14 hours in domestic chicken. The Pied crow (*Corvus albus*) was less sensitive with no signs of toxicity at 10 mg/kg and a plasma half-life of 2.5 hour (Naidoo *et al*., 2007, Naidoo *et al*., 2010).

Due to inter-species sensitivity and the apparent relationship between the plasma half-life of elimination, it was suggested that the lethal effect of the NSAIDs in avian species is associated to their ability to metabolise the drug in species-specific manner, with limitation being present at the domain of the cytochrome P450 (CYP450) enzyme network, which is responsible for xenobiotic metabolism. Naidoo *et al* (2010) postulated that toxicity in vultures was due to zero-order metabolism related to a possible evolutionary pharmacogenetic defect in the CYP2C family resulting in non-expression of the enzyme system, based on similar effects in human with metabolic defects in the same enzyme family (Naidoo *et al*., 2010). At present the CYP enzyme of the vulture is yet to be identified. However with CYP enzymes sharing an evolutionary link (Bort *et al*., 1999, Goodman and Gilman, 2011, Watanabe *et al*., 2013), we speculate that species susceptible to toxicity would share evolutionary relationship, which should be visible in the mitogenome (Yang, Ye & Huang, 2016). For this study the complete mitogenomes were used as surrogates for these comparisons.

1.3 Materials and Methods

1.3.1 Materials

Sodium pentobarbital (Euthapent®), ZR Genomic DNA Isolation kit (Zymo Research), BigDye Terminator sequencer Cycle Sequencing Kit, oligonucleotide primers (Integrated DNA Technologies), liquid nitrogen (Afrox), 2 ml cyrotubes (Greiner Bio-One, Frickenhausen) were used in the study. The equipment used for the study were -80°C refrigerator, NanoDrop spectrophotometer (Thermo Fisher Scientific), microcentrifuge (Eppendorf), ION Torrent S5 (Thermo Fisher Scientific) Next Generation Sequencer, 540 ION chip (Thermo Fisher Scientific),

ABI 3130 Genetic Analyser (Applied Biosystems) and SimpliAmp Thermal Cycler (Thermo Fisher Scientific).

1.3.2 Methods

1.3.2.1 Publicly available sequence information

The complete mitochondrial genomes of the bird species used for these studies were obtained from GenBank and they belong to the following families namely: Accipitridae, Pandionidae, Sagittariidae, Cathartidae, Falconidae and Strigidae respectively (Table 1).

1.3.2.2 Collection of Skin Samples and Genomic DNA Extraction

The bird species used during this study was authorized by the Animal Ethics Committee of the University of Pretoria, South Africa, with project numbers V093-15 and V097-17. Samples were opportunistically collected immediately after the euthanasia of an individual Cape vulture (*Gyps coprotheres*) with intravenous injection of pentobarbitone for a non-treatable physical injury. Skin samples were stored in sterile cryotubes and placed immediately into liquid nitrogen (-196°C) for 10 minutes to snap-freeze the samples, which were then transferred into the -80°C refrigerator until genomic DNA extraction. The frozen Cape vulture skin samples were allowed to melt at ambient temperature and approximately 25 mg of the thawed tissue was excised for DNA extraction using the ZR Genomic DNA Isolation kit (Zymo Research) according to manual instructions. The quality of the extracted sample was examined using a NanoDrop reader. In addition, blood samples were collected from six unrelated Cape vulture in polycarbonated heparinised tubes for PCR amplification of COX1, COX3 and NAD3 genes. DNA extraction and assessment of the DNA quality were similar to the above-mentioned protocol for the skin sample.

1.3.2.3 Genome sequencing

Genome sequencing was performed at the Uppsala Genome Centre, Uppsala University, Uppsala, Sweden, on the ION S5 XL platform (Thermo Fisher. 2015). The genome sequencing was conducted according to manual instruction. The run was performed on 200bp read length chemistry on an ION-540 chip. ~~The 540 chip contains 8 barcoded chips for sample tracking and sequencing. It electronically detects polymerase driven base incorporation without the use of fluoresence with a rapid time run of 2.5 hours and generating 60-80 million reads~~.

1.3.2.4 Mitogenome assembly, annotation and PCR amplification and sequencing

The NGS sequence quality was evaluated using the FastQC software (Bioinformatics, 2011). Based on the quality assessment the data was trimmed using the Trimmomatic program (Bolger *et al.,* 2014) and the total dataset was down sampled to 10 million reads and reads with lengths exceeding 100bp were selected for subsequent assembly. The de-novo assembly was conducted using the CLC Genomic Workbench version 6.0 software on the default settings. The subsequent assemblies were used to create a database against which a representative mitochondrial genome was queried. The contig with significant similarity to the query across its entire length was then submitted to the MITOS server (Bernt *et al*., 2013) in order to perform annotation of the mitochondrial genome. In addition, amplification of three mitogene (COX1, COX3 and NAD3) regions was performed using primers designed based on the assembled mitogenome as reference. The regions that were interrogated are identified as those genes for which a stop codon could not be identified (COX3), as well as the genes that contained putative frameshifts (COX1 and NAD3) based on initial MITOS annotation of the assembled mitogenome. The PCR output was evaluated using 1% agarose gel electrophoresis. The purified PCR samples were sequenced on the ABI 3130 Genetic Analyser (Applied Biosystems) using the BigDye Terminator sequencer Cycle Sequencing Kit and gene specific primers for COX1, COX3 and NAD3. Additionally, to verify the absence of stop codon and presence of putative frameshift in COX1, COX3 and NAD3 mitogenes, these genes were also amplified and sequenced from six unrelated Cape vultures. Primers used for the amplification of these genes are presented in Table 2.

1.3.2.5 Mitogenome structure, organization and characterization of Gyps coprotheres

The Cape vulture mitogenome order, organization and characterization were described as presented in *Fig.1* and Table 3. Gene overlap and intergenic-space sequences were determined manually. The putative origin of light-strand replication (O_L) and control region were identified by comparison with the homologous sequences of other bird species from the Accipitriformes order.

1.3.2.6 Phylogenetics

The correlation between phylogeny and susceptibility to diclofenac toxicity was inferred using maximum likelihood analysis model in MEGA X between bird species included in this study (Hall, 2013). The raptor species included in the phylogenetic analysis belong to the Accipitridae, Falconidae, Strigidae and Cathartidae families while *Strix leptogrammica w*as used as an outgroup (*Fig.2*). To determine the evolutionary relationship between phylogeny and susceptibility to diclofenac toxicity among the raptor bird species, each bird species complete mitogenome was analysed using the maximum likelihood model in MEGA X. Preference model analysis was conducted on the aligned nucleotide sequences on MEGA X maximum likelihood algorithm and GTR+G+I was determined to be the best model for constructing the phylogenetic tree. Phylogenetic trees were constructed using this model with 1000 bootstrap replicates according to the protocol described by Hall Berry G (2013) (Hall, 2013).

1.4 Results

1.4.1 Mitogenome structure, organization and characterization of Gyps coprotheres

The Cape vulture mitogenome is a 16908bp circular DNA molecule with 13 protein coding genes (PCGs), 22 transfer RNA (tRNA), 2 ribosomal RNA (rRNA) and a non-coding region known as the D-loop (GenBank accession no.MF683387; *Fig.1*, Table 2). The most used start codon is ATG with 76.92% frequency while ATA, GTG and ATT were alternate initiation codons. However, for termination codon TAA was the most used with 53.85% frequency while AGG and TAG served as alternate stop codon. Furthermore, NAD4 and COX3 do not have stop codons, but had "T" as their last nucleotide (Table 3). The architecture of the Cape vulture was similar to those of the raptor bird species included in this study (Jiang *et al.,* 2019).

1.4.2 PCR amplification and sequencing

Sanger sequencing of the COX1 gene identified a missing "C" nucleotide at position 6965 in the NGS annotated mitogenome, which was not present in the original mitogenome assembled. This error is a common phenomenon associated with next generation sequencing of homo-polymeric regions (Buermans, Den Dunnen, 2014). Furthermore, the insertion of this missing "C" nucleotide at position 6965 of the NGS annotated mitogenome resolved the frame

229 shift mutation earlier predicted by the MITOS annotation observed in COX1 and NAD3
230 mitogenes.

231 Sanger sequencing confirmed the absence of a stop codon in COX3 in the Cape vulture
232 sample that was used for NGS as well as in the additional six Cape vultures for which the COX3
233 gene was sequenced. These birds all had "T" as their last nucleotide in COX3 gene. The same
234 phenomenon was also observed in all the raptor bird species included in this study (Table 1).
235 Previous studies report that bird species were shown to polyadenylate their last T nucleotide in
236 mRNA to generate a stop codon (Slack *et al*., 2007, Doyle *et al*., 2014). This implies that the
237 Cape vulture may also generate a stop codon for COX3 and NAD4 mitogenes by
238 posttranscriptional polyadenylation of the last T nucleotide in mRNA (Slack *et al*., 2003).

239 **1.4.3 Phylogenetic analyses**

240 For this study, the correlation between phylogeny and susceptibility to diclofenac toxicity
241 were investigated using complete mitogenome among all the raptor species whose complete
242 mitochondrial genome are available in GenBank. The choice of the mitogenomes as surrogates to
243 investigate correlation between phylogeny and susceptibility is because the mitogenome is
244 composed of unique DNA sequences with evolutionary characteristic among animal species
245 (Jiang et al., 2015, Jiang et al 2019).

246 **1.5 Discussion**

247 The phylogenetic analyses inferred from the complete mitogenome sequences among the
248 raptor bird species included in this study indicated a monophyletic relationship among the
249 Accipitridae. With the Buteo+Butastur and Accipiter+Circus genera clustering together as a
250 clade. While the Gyps+Aegypius+Spilornis and Nisaetus+Aquila genera forming a clade. The
251 tree showed close phylogenetic relationship among the Gyps vultures. Furthermore, the Gyps
252 genus have close relationship to Aegypius followed by Spilornis, Nisaetus, and Aquila genera
253 respectively. The Pandion genus had close relationship to the Accipitridae family followed by
254 Sagittarius, Cathartes and the Falco genera (Johnson *et al*., 2016). The result showed a
255 monophyletic relationship among Falcons which are closely related to the Accipitridae family
256 compared to the Owl. The hierofalcons (*Falco cherrug* and *Falco rusticolus*) and *Falco*
257 *peregrinus* cluster together into one clade with 100% bootstrap support value. Furthermore,

Falco tinnunculus and *Falco naumanni* had a monophyletic relationship with 100% bootstrap value while *Falco columbarius* and *Falco sparverius* are outgroups. This study further confirmed the monophyletic relationship existing among Falcons (Helbig *et al.,* 1994, Wink *et al.,* 2004, Nittinger *et al.,* 2005, Doyle *et al.,* 2018).

Due to earlier pharmacokinetics studies of NSAIDs in Old World vulture and other bird species (Swan *et al*., 2006b, Naidoo *et al*., 2010, Naidoo *et al*., 2017), there is a species-specific relationship associated to NSAIDs toxicity among bird species (Rattner *et al*., 2008, Naidoo *et al*., 2009a, Naidoo *et al*., 2010, Naidoo *et al*., 2011, Naidoo *et al*., 2017). It is also known that all Old World vultures are vulnerable to the lethal consequences of diclofenac with the exception of New World vulture, pied crow and to some extent domestic chicken which are susceptible at higher concentration (Table 1) (Rattner *et al*., 2008, Naidoo *et al*., 2009a, Naidoo *et al*., 2010, Naidoo *et al*., 2011). In addition, the detrimental consequences of diclofenac have also been reported in the steppe eagle which is a descendant of the Aquila genus (Sharma *et al*., 2014).

The phylogenetic analysis results (*Fig.2*) clustered together the Old world vultures (Gyps and Aegypius genera), Hawks (Nisaetus genus) and Eagles (Spilornis and Aquila genera) in the same clade.. Considering the susceptibility of Old World vultures and one Eagle (*Aquila nipalensis*) species to diclofenac toxicity from previous studies (Naidoo *et al*., 2017, Sharma *et al*., 2014), it can be deduced that there is a correlation between phylogeny and susceptibility to diclofenac toxicity as indicated in the phylogenetic analysis (*Fig. 2*). The possibility thus exists that members of the Hawk and Eagle families succumbing to diclofenac toxicity would be higher due to their close evolutionary relationship to the Old World vultures which are known to be susceptible to NSAIDs toxicity (Swan et al., 2006, Naidoo et al., 2007, Naidoo et al., 2017) than the other members of the Accipridae. However hawks and eagles may be somewhat more protected as a result of their feeding habits which solely rely on hunting and less commonly on carrion feeding as opposed to the old vultures which are purely carrion feeders i.e. the opportunity to be exposed to medicated carrions is higher for the vultures.

The results would also tend to suggest that the falcons, swls, harries, bustards, buzzards, kestrels and hawks would be less likely susceptible to diclofenac, since they are an out grouping as seen with the Turkey vulture which is resistant to diclofenac at a dose that is 100 times the lethal dose seen in Old World vultures.

The reason for a shared susceptibility among Accipitridae may be explained by evolutionary changes in the cytochrome P450 (CYP) group of enzymes. As group of enzymes they are important in the detoxification of environmental pollutants and xenobiotics (Bort *et al*., 1999, Goodman and Gilman, 2011, Watanabe *et al*., 2013). It thus stands to reason that with the mitogenome indicating species similarity; these species would evolve under similar environmental conditions and thus developed similar CYP enzyme capacity. The latter was demonstrated with the cholinesterase enzyme system whereby the concentrations in herbivores is naturally higher than carnivores, due to plants having higher concentrations of natural acetyl choline like substances in comparison to animals (Ruiz-Garcia *et al*., 2008). As a result, the evolutionary adaption of higher enzyme concentration of these enzyme results in herbivores being less susceptible to organophosphorus toxicity (Ruiz-Garcia *et al*., 2008).

Evolutionary variations in the CYP450 enzymes between diclofenac resistant bird species (Turkey vulture, Pied crow, Chicken) and susceptible Old World vultures can better explain species-specific toxicity observed among avian. It is therefore imperative to identify members of the CYP450 group of genes in birds to fully elucidate the reason behind resistance and susceptibility to NSAIDs.

1.6 Conclusion

The architecture of the Cape vulture mitogenome was similar to the raptor bird species included in this study. The mitogenome suggests a correlation between phylogeny and susceptibility to diclofenac toxicity among Old world vultures, Hawks, Eagles and other members of the Accipitridae family. The Accipitridae susceptibility to diclofenac toxicity may be suggestive of evolutionary changes in the CYP genes responsible for xenobiotic metabolism.

1.7 Acknowledgements

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1.8 References

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Figure 1

Complete mitochondrial genome organization and mitogene arrangement of *Gyps coprotheres*.

Genes found on the coding strand are indicated outside the mitochondrial genome map, while the mitogenes coded on the complementary strand are indicated inside the map.

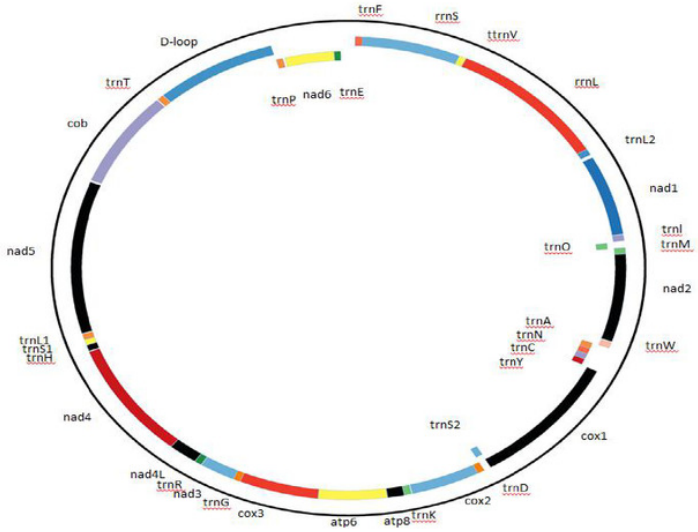
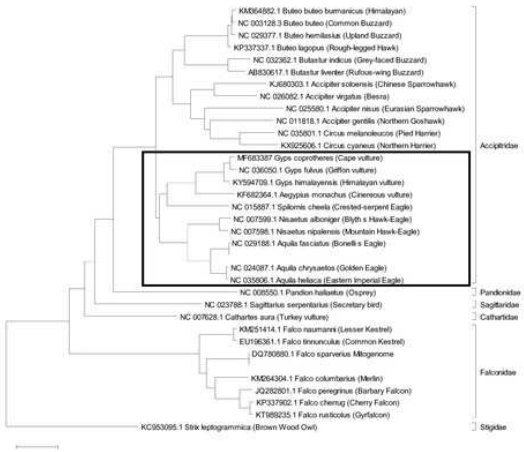

trnF
rrnS
ttrnV
rrnL
trnL2
nad1
trnI
trnM
nad2
trnW
cox1
trnD
cox2
trnK
atp8
atp6
cox3
trnG
nad3
trnR
nad4L
nad4
trnH
trnS1
trnL1
nad5
cob
trnT
D-loop
trnP
nad6
trnE
trnS2
trnY
trnC
trnN
trnA
trnO

Figure 2

Phylogenetic Analysis Result using complete mitogenome

Results of Phylogenetic analyses using maximum likelihood (ML) analysis indicated evolutionary relationships among 33 raptor species based on complete mitogenome sequences. *Strix leptogrammica* was used as outgroup. Bootstrap support values for ML analyses are indicated on the nodes. The solid border rectangle indicates a close phylogenetic relationship between Old World vultures, Hawk and Eagles confirmed with high bootstrap values with greater chances of shared susceptibility based on their close evolutionary relationship. While distantly related raptor bird species are less likely to succumb to diclofenac toxicity as reported for Turkey vulture (*Cathartes aura*) (Rattner et al., 2008)



Falco columbaris – Merlin Falcon??

Falco sparverius – Needs a common name

Buteo buteo burmanicus – Himalayan buzzard??

Table 1(on next page)

Mitogenome accession number and Diclofenac Toxicity Status of Bird Species investigated in this study as classified by Clements et al (2019).

(A) Bird species names, (B) Family name, (C) Accession number, (D) Genera Diclofenac toxicity status, (E) References

Species	Family	Accession number	Genus Diclofenac Toxicity Status	References
Accipiter gentilis (Northern Goshawk)	Accipitridae	NC_011818	Suspected	N/A
Accipiter nisus (Eurasian Sparrowhawk)	Accipitridae	NC_025580	Suspected	N/A
Accipiter soloensis (Chinese Sparrowhawk)	Accipitridae	KJ680303	Suspected	N/A
Accipiter virgatus (Besra)	Accipitridae	NC_026082	Suspected	N/A
Aegypius monachus (Cinereous Vulture)	Accipitridae	KF682364.1	Susceptible	Ogada *et al* 2011
Aquila chrysaetos (Golden Eagle)	Accipitridae	NC_024087	Susceptible	Sharma *et al* 2014
Aquila fasciata (Bonelli's Eagle)	Accipitridae	KP329567	Susceptible	Sharma *et al* 2014
Aquila heliacal (Eastern imperial eagle)	Accipitridae	NC_035806	Susceptible	Sharma *et al* 2014
Buteo buteo (Common Buzzard)	Accipitridae	NC_003128	Suspected	N/A
Buteo buteo burmanicus (Himalayan)	Accipitridae	KM364882	Suspected	N/A
Buteo fasciatus (Bonelli's eagle)	Accipitridae	NC_029188	Suspected	N/A
Buteo hemilasius (Upland Buzzard)	Accipitridae	NC_029377.1	Suspected	N/A
Buteo lagopus (Rough-legged Hawk)	Accipitridae	KP337337	Suspected	N/A
Butastur indicus (Grey-faced Buzzard)	Accipitridae	NC_032362	Suspected	N/A
Butastur liventer (Rufous-wing Buzzard)	Accipitridae	AB830617	Suspected	N/A
Cathartes aura (Turkey Vulture)	Cathartidae	NC_007628	Not susceptible	Rattner et al 2008
circus cyaneus (Northern Harrier)	Accipitridae	KX925606	Suspected	N/A
circus melanoleucos (Pied Harrier)	Accipitridae	NC_035801	Suspected	N/A
Gyps coprotheres (Cape vulture)	Accipitridae	MF683387	Susceptible	Niadoo et al 2007
Gyps fulvus (Griffon vulture)	Accipitridae	NC_036050	Susceptible	Ogada et al 2011

Gyps himalayensis (Himalayan vulture)	Accipitridae	KY594709.1	Susceptible	Ogada et al 2011
Nisaetus alboniger (Blyth's Hawk-Eagle)	Accipitridae	NC_007599	Suspected	N/A
Nisaetus nipalensis (Mountain Hawk-Eagle)	Accipitridae	NC 007598.1	Suspected	N/A
Spilornis cheela (Crested Serpent-Eagle)	Accipitridae	NC_015887	Suspected	N/A
Sagittarius serpentarius (Secretary-bird)	Sagittariidae	NC_023788	unknown	unknown
Pandion haliaetus (Osprey)	Pandionidae	NC_008550	unknown	unknown
Strix leptogrammica (Brown wood owl)	Strigidae	KC953095.1	unknown	unknown
Falco columbarius (Merlin)	Falconidae	KM264304.1	unknown	unknown
Falco tinnunculus (Common Kestrel)	Falconidae	EU196361.1	unknown	unknown
Falco sparverius (American kestrel)	Falconidae	DQ780880.1	unknown	unknown
Falco naumanni (Lesser Kestrel)	Falconidae	KM251414.1	unknown	unknown
Falco peregrinus (Barbary falcon)	Falconidae	JQ282801.1	unknown	unknown
Falco cherry (Cherry Falcon)	Falconidae	KP337902.1	unknown	unknown
Falco rusticolus (Gyrfalcon)	Falconidae	KT989235.1	unknown	unknown

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Table 2(on next page)

Primers used for PCR amplification of COX1, COX3 and NAD3 mitogenes

(A) COX1 Forward and Reverse Primer Sequences, (B) COX3 Forward and Reverse Primer Sequences, (C) NAD3 Forward and Reverse Primer Sequences

Mitogene	Forward Primer Sequences	Reverse Primer Sequences
COX1	5’-CGC CTA CAC CCT ATG AAA TAC, C-3	5’-TAT AGG ACT AGG CTG CAG ATG G-3’
COX3	5’-AGC TGC CTG ATA CTG ACA CTT C-3’	5’-AGT AAG TGA GTT CGG TGG AAG G-3’
NAD3	5’-TGG GTC ATC CTT CCT ATC AGT C-3’	5’-AGT GAC ATG GAG AGA GGC ATA G-3’

1

Table 3(on next page)

Characteristics of the mitochondrial genome of *Gyps coprotheres*

(A) Mitogene names, (B) mitogene position, (C) Mitogene nucleotide size, (D) Mitogene start and stop codon, (E) Mitogene intergenic overlap, (F) Mitogene strand, (G) Mitogene A+T% nucleotide composition

Gene	Position		Size	Condon		Intergenic overlap	Strand	Nucleotide composition
	From	To	Nucleotide	Start	Stop			A+T%
tRNA-Phe	1	70	70			0	H	47.2
12S rRNA	70	1037	968			0	H	51.2
tRNA-Val	1037	1108	72			0	H	55.6
16S rRNA	1109	2709	1601			1	H	54
tRNA-Leu	2710	2783	74			1	H	47.3
ND1	2822	3800	978	ATG	AGG	39	H	53.9
tRNA-Ile	3769	3840	72			-31	H	55.5
tRNA-Gln	3854	3924	71			14	L	67.6
tRNA-Met	3924	3992	69			0	H	49.3
ND2	3993	5039	1047	ATG	TAG	1	H	52.6
tRNA-Trp	5038	5109	72			-1	H	62.5
tRNA-Ala	5111	5179	69			2	L	56.5
tRNA-Asn	5182	5254	73			3	L	50.7
tRNA-Cys	5257	5323	67			3	L	49.3
tRNA-Tyr	5324	5393	70			1	L	55.7
COX1	5395	6945	1551	GTG	AGG	2	H	52.8
tRNA-Ser	6937	7010	74			-8	L	52.7
tRNA-Asp	7015	7083	69			5	H	59.4
COXII	7086	7769	684	ATG	TAA	3	H	52.8
tRNA-Lys	7771	7841	71			2	H	59.1
ATP8	7843	8010	168	ATG	TAA	2	H	55.6
ATP6	8001	8684	684	ATG	TAA	-9	H	54.8
COXIII	8684	9467	784	ATG	T	0	H	52.9
tRNA-Gly	9468	9536	69			1	H	66.6

ND3	9537	9710	351	ATT	TAA	1	H	55.1
tRNA-Arg	9893	9961	69			183	H	59.4
ND4L	9963	10259	297	ATG	TAA	2	H	53.9
ND4	10253	11630	1378	ATG	T	-6	H	51.3
tRNA-His	11631	11700	70			1	H	65.7
tRNA-Ser	11702	11766	65			2	H	55.4
tRNA-Leu	11767	11837	71			1	H	62.0
ND5	11847	13652	1806	ATA	TAA	10	H	55.2
Cytb	13665	14807	1143	ATG	TAA	13	H	52.3
tRNA-Thr	14810	14877	68			3	H	64.8
tRNA-Pro	16083	16152	70			1206	L	61.4
ND6	16174	16692	519	ATG	TAG	22	H	50.3
tRNA-Glu	16693	16763	71			1	L	62.0
D-loop	14878	16082	4			-1885	H	58.6
Unknown Region	16764	16908	145			682	H	62.7

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