The Complete Mitochondrial Genome of Gyps coprotheres (Aves, Accipitridae, Accipitriformes): A correlation between phylogeny and susceptibility to diclofenac toxicity among raptors

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1.1 Abstract

Three species of ~~old~~ Old ~~world~~ World vultures on the Asian peninsula are slowly rejuvenating from the lethal consequences of diclofenac. At present the reason for species sensitivity is unknown. Further it has since been demonstrated that other ~~old~~ Old ~~world~~ World vultures like the Cape (*Gyps coprotheres*) (CGV) and griffon (*G. fulvus*) vultures are also susceptible. Oddly the new world Turkey vulture (*Cathartes aura*) and Pied crow (*Corvus albus*) are not susceptible. 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 mitogene, specifically using the 13 protein-coding (13PCG) and Cytochome C oxidase (COXI) mitogenes. This was achieved by the 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 COX1 phylogeny and susceptibility among old world vultures, Aquila eagle species and other members of the Accipitridae family. Susceptibility thus appears to be due to evolutionary reasons.

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

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1.2 INTRODUCTION

Generally, vultures may be classified as old or new world vultures based on the apparent convergent evolutionary scavenging feeding habit. However, in reality, they are morphologically and evolutionary diverse group of birds (Seibold, Helbig, 1995, Wink, 1995, Johnson *et al.*, 2016)~~.~~. Old ~~world~~ World vultures are descendants of the Accipitridae family comprising of eagles, hawks, kites and buzzards. According to Clements taxonomic classification, Accipitridae is one of the largest non-passerine members of bird species comprising of 247 species (64 are monophyletic while 25 are polyphyletic) belonging to the order Accipitriformes (Clements *et al.*, 2017). 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.*, 2017).

Raptors are birds of prey 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.*, 2017). 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 in India, Nepal and Pakistan from the consumption of carcasses from animals dosed with diclofenac before dying (Adawaren *et al.*, 2018, Oaks *et al.*, 2004, Swan *et al.*, 2006b, Naidoo *et al.*, 2009a). 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 (Naidoo *et*

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al., 2017, Adawaren *et al*., 2018, Swan *et al*., 2006b, Naidoo *et al*., 2009b, Naidoo *et al*., 2010)

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 ~~characterised~~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 characterized 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~~ Old ~~world~~ World bird species are also vulnerable to other NSAIDs toxicity with the notable exception of meloxicam thus far (Adawaren *et al*., 2018, Swan *et al*., 2006a, Naidoo *et al*., 2010). The scenario is, however, different with the new world vultures. In a study in ~~which the~~which the turkey vulture (*Cathartes aura*) was exposed to concentration of diclofenac (25 mg/kg) 100 ~~times above~~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 (Naidoo *et al*., 2009a, Rattner *et al*., 2008). The high sensitivity of the ~~old world~~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*., 2010, Naidoo *et al*., 2007).

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 mitogenome of the 13 coding mitogenes were used as surrogates for ~~these comparison~~these comparisons.

1.3 Materials and Methods

1.3.1 Materials and Equipment

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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~~ 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).

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1.3.2.2 Collection of Skin Samples and Genomic DNA Extraction

The bird species used during this study was ~~authorised~~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~~above-mentioned protocol for the skin sample.

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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.

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, Lohse & Usadel, 2014) and the total dataset was down sampled to 10 million reads and reads with lengths exceeding 100_bp 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.

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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.2-4*). To determine the evolutionary relationship between phylogeny and susceptibility to diclofenac toxicity among the raptor bird species, 13 PCG, Cytb, and COXI mitogenes from each bird species mitogenome were ~~analysed~~analyzed 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).

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1.4 Results and Discussion

1.4.1 Mitogenome structure, organization and characterization of Gyps coprotheres

The Cape vulture mitogenome is a 16908 bp 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.

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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 frameshift mutation earlier predicted by the MITOS annotation observed in COX1 and NAD3 mitogenes.

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

1.4.3 Phylogenetic analyses

For this study, the correlation between phylogeny and susceptibility to diclofenac toxicity were investigated using 13 PCGs, Cytb and COX1 among all the raptor species whose complete mitogenome are available in GenBank. The choice of these genes as surrogates to investigate correlation between phylogeny and susceptibility were based on the following reasoning. Cyclooxygenase 1 (COX1) was used because of its involvement in the adverse and toxic effects of non-steroidal anti-inflammatory drugs (NSAIDs) and cell death (a described pathological change of the proximal convoluted tubules in vultures on necropsy) while cytochrome b (Cytb) mitogene has been used in previous studies to establish phylogenetic relationship among different animal species (Lerner, Mindell, 2005, Meteyer et al., 2005, Jiang et al., 2015). In addition, the Cytb mitogene of the steppe eagle (*Aquila nipalensis*, GenBank Accession no. AY987287.1) was included in the phylogenetic analysis as this eagle has been shown to succumb to diclofenac toxicity. All 13 PCGs were included to avoid bias due to the use of single mitogene (Jiang *et al.*, 2015).

The phylogenetic analyses inferred from COXI gene indicate a monophyletic relationship among members of the Accipitriformes order (*Fig.2*). Similar monophyletic relationships were observed among the Falconidae family (*Fig.2*). In addition, the phylogeny indicates a close relationship between ~~old~~ Old ~~world~~ World vultures and eagles (Aquila species) (*Fig.2*). The Old World Vulture (Gyps and Aegypius) + Eagle clades-Nisaetus clades indicate a close monophyletic relationship to Buteo-Butastur + Accipiter-Circus clades with *Pandion haliaetus* and *Sagittarius serpentarius* branching out as sisters. The former ~~indicate~~indicates a close relationship

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to the Accipitridae followed by the latter. However, the Falconidae, Cathartidae and Stigidae are distantly related to the Accipitriformes (*Fig.2*).

Furthermore, the phylogenetic analysis of 13 PCG sequences among the raptor bird species included in this study, showed a similar pattern to that observed with COX1 gene (*Fig.2 and 3*). However, *Sagittarius serpentarius* and *Pandion haliaetus* indicated a monophyletic relationship even though they belong to different families. This could possibly be as a result of the convergence of COX1 gene in both species, even though they belong to different families (Johnson *et al.*, 2016). In addition, the 13 PCG phylogenies indicated a close evolutionary relationship between old world vultures and eagle (Aquila) species (*Fig.2 and 3*).

In view of the ~~above mentioned~~above-mentioned evolutionary relationship, inferences drawn from cytochrome b (Cytb) phylogenetic tree also showed a close relationship between ~~old~~ Old ~~world~~ World vultures ~~and eagle~~and eagle (Aquila) species (*Fig.4*). Furthermore, *Sagittarius serpentarius* and *Pandion haliaetus* are distinctly separated (as shown in COX1 tree *Fig.2*) with no monophyletic relationship indicated by 13 PCG (*Fig.3*). This clearly showed that both species belong to different families and the use of more than one mitogenes is important in resolving evolutionary differences among closely related species (Johnson *et al.*, 2016).

Due to earlier pharmacokinetics studies of NSAIDs in old world vulture and other bird species (Naidoo *et al.*, 2017, Swan *et al.*, 2006b, Naidoo *et al.*, 2010), there is a species-specific relationship associated to NSAIDs toxicity among bird species (Naidoo *et al.*, 2017, Naidoo *et al.*, 2009a, Naidoo *et al.*, 2010, Rattner *et al.*, 2008, Naidoo *et al.*, 2011). 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 (Naidoo *et al.*, 2009a, Naidoo *et al.*, 2010, Rattner *et al.*, 2008, 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.2-4*) shows a close relationship between old world vultures and eagles (Aquila species). Considering the susceptibility of both 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. The possibility of any member of the Aquila eagle species succumbing to diclofenac toxicity would be higher.

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However, the absence of diclofenac toxicity among the Aquila species might likely also be as a result of their feeding habits which solely rely on hunting, rather than feeding on medicated carrions. In addition, there are high possibilities of toxicity among members of the Accipitridae family due to their close evolutionary relationship to the ~~old~~ Old ~~world~~ World vultures known to be susceptible to NSAIDs toxicity. The results would also tend to suggest that the falcons, owl, and turkey vulture would be unlikely susceptible to diclofenac, and may explain why no reports of toxicity has been reported for these species.

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 mitogene 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).

1.5 Conclusion

The architecture of the Cape vulture mitogenome was similar to the raptor bird species included in this study. The COX1 gene suggests a correlation between phylogeny and susceptibility to diclofenac toxicity among old world vultures, eagles and 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.

1.6 Acknowledgements

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