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Genetic polymorphism in the mitochondrial D-loop of Oriental White-backed Vultures *(Gyps bengalensis)*

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Abstract Vultures are among nature's most successful scavengers, providing tractable models for ecological, economic, and cultural studies. Asian vultures have undergone dramatic declines of 90–99% in the subcontinent due to consequences of poisoning drugs, thereby being at a high risk of extinction. In Pakistan, surveys conducted previously focused mostly the cause of decline and breeding strategies only. Genetic profiling of vultures was still unmapped that could play a particular role in conservation endeavors and let researchers to genetically label individuals of threatened or endangered species. In this study, we examined genetic diversity and molecular phylogeny of Oriental White-backed Vultures by analyzing mitochondrial DNA (mtDNA) sequences. Genetic polymorphism was detected among individuals, and, on that basis, phylogenetic analysis was conducted through Bayesian analysis of DNA sequences using MCMC. Using multiple sequence alignment, two mutations, transversion T>G and transition G>A, were observed at nucleotide positions 1 and 2, respectively. Similarly, T/C heterozygosity at two positions, 53 and 110, and one heterozygous T/G locus at 130 position were also observed. The reference sequence, along with other samples of V1, V6, V7 and V9, was placed into a clade, while V2, V5, V11, V3, V4 and V10 samples were grouped into a two clade.

Keywords: vulture, *Gyps bengalensis*, phylogenetics, mitochondrial D-loop, Pakistan

Összefoglalás A keselyűk a természet legsikeresebb dögevői közé tartoznak, jól nyomon követhető modellt szolgáltatva ökológiai, gazdasági és kulturális kutatásokhoz. Az ázsiai keselyűpopulációk drámai, 90–99%-os csökkenésen mentek keresztül a szubkontinensen a gyógyszerek használatából fakadó mérgezések miatt, így magas a kihalásuk veszélye. Pakisztánban a korábban végzett vizsgálatok leginkább az állománycsökkenés okára és a költési stratégiák felderítésére összpontosítottak. A keselyűk genetikai profiljának feltérképezése még mindig nem történt meg, ami nagy szerepet játszhatna a konzervációs törekvésekben, valamint ezáltal a kutatóknak lehetőségük nyílna a veszélyeztetett fajok egyedeinek genetikai jelölésére. Ebben a tanulmányban a bengál keselyű genetikai diverzitását és molekuláris filogenetikáját vizsgáltuk mitokondriális DNS (mtDNS) szekvenciák elemzésével. Az egyedek között genetikai polimorfizmus volt megfigyelhető, így a DNS-szekvenciák alapján filogenetikai analízist végeztünk Bayesi módszerrel MCMC-t használva. Többszörös szekvencia-illesztést alkalmazva két mutációt, a T>G transzverziót és a G>A tranzíciót figyeltük meg az 1. és 2. nukleotid pozíciókban. Ugyanígy két pozíció T/C heterozigóta volt az 53. és a 110. helyen, valamint egy lókusz volt heterozigóta T/G a 130. pozícióban. A referenciaszekvencia a mi V1, V6, V7 és V9 mintáinkkal együtt egy kládba, míg a V2, V5, V11, V3, V4 és V10 mintáink egy másik kládba tartoztak.

Kulcsszavak: keselyű, bengál keselyű, *Gyps bengalensis*, filogenetika, mitokondriális D-hurok, Pakisztán

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Introduction

Vultures (Family Accipitridae) are scavengers with hunting strategies, thereby playing important ecological roles in maintaining ecosystem balance and aesthetic roles in environmental cleaning by feeding on animal's dead bodies, as well as controlling diseases spread by removing animal's carcasses. At a single time, vultures can consume more than 20% of their total body mass, therefore vulnerable to poisoning, particularly at carcasses that are laced with poison. Vultures are blessed with a unique digestive system that holds distinctive acids to dissolve bacterial strains of cholera anthrax, botulism and anthrax (Wink 1995). Mainly two taxonomic groups of vultures are known to date, and they fall into two broad categories: Old World and New World Vultures. They are the only vertebrate foragers but experienced the most rapid decline among birds over the past decades, which ranked them as one of the most threatened avian functional guilds in Asia and Africa (Buechley *et al.* 2016). Locally, eight species are present among these birds, and about 96% of decline in population of *Gyps bengalensis* was recorded in different areas of Pakistan, India, and Nepal during periods of 1991–1993 (Prakash 1999, Gilbert *et al.* 2002, 2004).

The main reason behind the decline of vulture's species is the unintentional poisoning of toxic NSAID (non-steroidal anti-inflammatory drug) Diclofenac by its veterinary use on cattle. Unfortunately, this catastrophic event continued unchecked for about 15 years due to usage of this anti-inflammatory drug (used to reduce the pain, fever and inflammation in livestock's) that emerged as the sole cause of rapid decline of Oriental White-backed Vulture population in Punjab, Pakistan (Oaks *et al.* 2001, Naidoo & Swan 2009). Vultures exposed to diclofenac during feeding on carcasses of livestock dozed with the drug and experienced kidney failure within a few days of their exposure (Swan *et al.* 2006). It has also been established that not only diclofenac but some other non-steroidal anti-inflammatory drugs (e.g. aceclofenac, carprofen, flunixin, ketoprofen, nimesulide and phenylbutazone) are also harmful to vultures and other scavenger birds (Cuthbert *et al.* 2006, Acharya *et al.* 2009). Since 2006, this drug has been proscribed by the administration of Pakistan, India and Nepal (Cuthbert *et al.* 2011). Although meloxicam is a single NSAID that was confirmed as a harmless drug designed for vultures (Harris 2013). Whereas further aspects that contribute to decline in vulture's populations include lack of food locally, habitat loss, severe climatic conditions, as well as poisoning via application of insecticides (Choisy 2013).

Information on the evolutionary history and genetic diversity of these species is considered critical for the success of both ex-situ and in-situ conservation. Mitochondrial DNA (mtDNA) of vultures was used due to some matchless properties, *i.e.* high copy number of mitochondria,

inheritance from mother only, absence of recombination and increased mutation rate. Poulakakis *et al.* 2008 studied the phytogeography of Black Vultures *(Aegypius monachus)* in Europe based on microsatellite and mtDNA variation. Few avian conservation studies have used SNPs so far García and Arruga 2006 used SNPs to differentiate species of partridges for reintroduction, and Väli *et al.* 2010 used them to examine hybridization between two species of Spotted Eagles in Europe. Clearly, the future is upon us, given the power of high-throughput sequencing and the associated power utility of SNPs (Susan *et al.* 2011).

The White-tailed Eagle *(Haliaeetus albicilla)* is used as a major flagship and umbrella species for conservation work throughout large parts of Europe. Loss of genetic diversity can reduce both short-term viability and long-term adaptability (Frankham 2005). To assess the genetic impact of population declines during the twentieth century, we therefore studied the genetic variability at mitochondrial DNA (mtDNA) control region sequences and autosomal microsatellite markers in north and central European white-tailed eagle populations (Hailer *et al.* 2006).The Griffon vulture population in Serbia, similarly to many Balkan countries, experienced a rapid demographic decline starting from the mid-twentieth century, mainly due to the mass poisoning of the birds and the implementation of new veterinary measures that prohibited the deposition of dead animals in nature (Marinkovic & Karadzic 2008). Genetic diversity analysis is of great importance in modern-day conservation, and without knowing the genetic status of the population it is hard to implement proper conservation measures and secure long-term survival of the total population in the nowadays fast-changing environment and habitat fragmentation (Maudetr *et al.* 2002).

Population declines of *Gyps* vulture species across south Asia have been well-documented since they were first reported in 1999 (Prakash 1999, Prakash *et al.* 2003, Gilbert *et al.* 2006). As a result of these declines, the Oriental White-backed Vulture *Gyps bengalensis*, Long-billed Vulture *Gyps indicus* and the Slender-billed Vulture *Gyps tenuirostris* are all listed as 'Critically Endangered' (Murn *et al.* 2015).

Although *Gyps* vulture populations were probably declining slowly in many parts of the world during the 20th century, a very different situation existed in India, Nepal and Pakistan. Three species of vultures endemic to South Asia, oriental White-backed Vulture, Long-billed Vulture *(G. indicus)* and Slender-billed Vulture *(G. tenuirostris)*, are listed as being threatened with extinction after rapid population declines in the Indian subcontinent, which began in the 1990s (Prakash *et al.* 2007). The Oriental White-backed Vulture population in India in 2007 was estimated at one-thousandth of its level in the early 1990s (Green *et al.* 2007).

In the Punjab Province of Pakistan, annual rates of decline for breeding populations of Oriental White-backed Vultures have ranged between 11% and 61% per year since 2001. Conservation initiatives to address the vulture declines have included the establishment and development of conservation breeding centres (Murn *et al.* 2008).

Six, out of nine species of vultures found in India have been facing problem of existence and therefore declared as threatened. Of these, three species endemic to South Asia, the Indian White-backed Vulture, Long-billed Vulture *(Gyps indicus)* and Slender-billed Vulture *(Gyps tenuirostris)* are at high risk of global extinction and are listed as critically endangered because of rapid population declines within the last decade in the Indian subcontinent (Thaku *et al.* 2012).

In this study, we analyzed genetic polymorphism of mitochondrial D-loop in *Gyps bengalensis* for phylogenetic relationship of this breeding population. Our objectives were to check molecular diversity in these populations, providing a basis for a more effective conservation effort for the recovery of globally threatened vultures. In Pakistan, WWF conservation deeds are appreciable in this regard, according to the current reports of SAVE Consortium (Saving Asia's Vultures from Extinction) Changa Manga Conservation Centre had seven pairs of this species of which four pairs attempted to breed. Depending on the genetic structure, it could potentially be the appropriate source population for future reintroduction programs.

Materials & Methods

Sample collection

Biological samples were collected from 11 Oriental White-backed Vultures Collection sites from Ex-situ conservation, vulture Captive Breeding Facility under Punjab Vulture Restoration and Conservation project at Changa Manga Forest, Kasur, Pakistan *(Figure 1)* after formal permissions from the Punjab Wildlife & Parks Department, Pakistan.

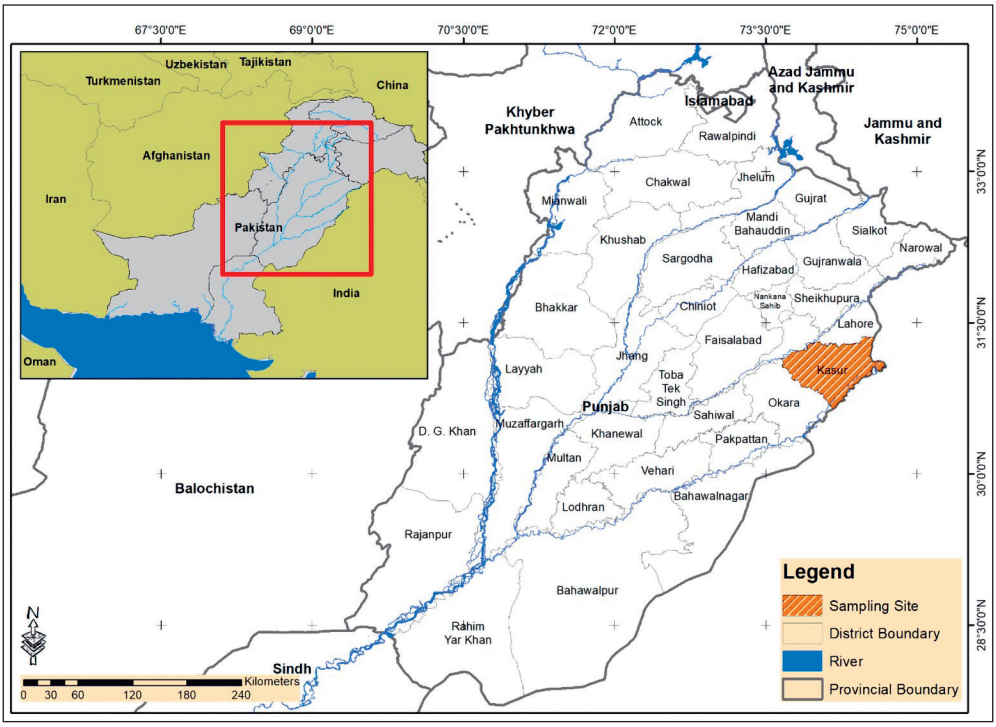

Figure 1. The samples collection sites from Punjab and Kasur
1. ábra A mintavételi helyek Punjab és Kasur tartományokban

DNA extraction

Genomic DNA was extracted from blood, by using protocol of Qiagen (QIAamp DNA Mini Kit, Cat No./ID: 51304). 1ml of blood samples was added in 350 µl Lysis Buffer (1 M Tris, 0.5 M EDTA, 5 M NaCl, 10% SDS), then centrifuged at 13.000 rpm for 5 min, and took pellet and repeated this step for 3 times at least, then added 40–50 µl of proteinase K and 70 µl of 10% SDS, centrifuged and took supernatant and added equal volume of phenol: chloroform: isoamyl alcohol (5:24:1), incubated for 10–15 min, centrifuged at 13,000 rpm for 3–5 min, and took supernatant.

Isopropanol (0.5 ml) was added to the samples, centrifuged again, then pellets were washed with 70% ethanol, and centrifuged again for 10 min. Samples were air dried for 12 hours. DNA samples were dissolved in 200 µl TE buffer or in DEPC-treated water. Then, a nanodrop method was applied at wavelength of 260/280 nm to check the purity and quantity of DNA samples (Sefc *et al.* 2003).

PCR amplification

Primers were designed for PCR amplification by Primer 3 program (Untergasser *et al.* 2012) We used the primers: GbCR4.L (5'-CGA TTC ATG GTA GCA GGT CA-3') and CSB1.H (5'-AAC ATG TCC AAC AAG CAT TCA-3') (Mullis *et al.* 1986). Next, extracted DNA was amplified by a subsequent PCR reaction (Mullis *et al.* 1986) in a total volume of 25 µl. PCR amplification consists of initial denaturation at 96 °C for 5 min, 30–35 cycles consisting of denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, then final extension at 72 °C for 10 min.

Sequencing and phylogenetic analysis

PCR samples were cleaned and washed with 70% ethanol and sent for sequencing by using a Beckman Coulter kit, and results were analyzed. More software's were used to analyze data including Codon Code Aligner Version 5.1.4 (CodonCode Corporation) for sequences alignment as well as for analysis, and BEAST v1.10.4 (Tamura *et al.* 2013) for phylogenetic analysis. Furthermore sequences were passed through BioEdit v7.0.9.1. (Hall 1999). Haplotype and nucleotide diversity were estimated using DNaSP v4.1 software (Librado & Rosaz 2009). Using the maximum parsimony (MP) method, we chose the tree having the highest likelihood value as our best from the NETWOK 4.5.1.2 (Bandelt 1999) and the species trees were reconstructed using the coalescent-based model implemented in BEAST v1.10.4 (Tamura *et al.* 2013). The two mtDNA loci were partitioned by codon for all analyses. The tree was constructed from 481 bp, using the first high variation section fragment (LVH). The reference sequence (previous sequence taken from GenBank) was used to derive a phylogenetic analysis to identity the haplogroup in the current isolate. Genetic variation data include demographic historical information. mtDNA of mismatch distribution (for comparison) is usually used to investigate demographic events (Slatkin & Hudson 1991, Rogers & Harpending 1992).

It has been developed two standards; not multimodal distribution rules must have a constant population size, and growing populations have a uniform single peak distribution.

Before the system phylogeny analysis, JModeltest 2.1.7 determined the appropriate DNA model analysis. In this analysis, the best model of HKY+1 was found for Neighbour connection parameters using the appropriate Bayesian information criteria. The original tree was analyzed by BioNJ using PhyML 3.1. Using a 100-start analysis to estimate the maximum probability node supports non-parametric pseudo-random. Bayesian analysis was implemented using MrBayes 3.2 (Geyer 1991). Finally, a random starting shirt was used for the analysis, yielding 10.000.000 generations for all species sequence data (100% of bootstrap support). It has been studied by every 100 generations frequently used to check the generation and transition by Markov Chain model. The pairwise difference matrix is performed using NETWOK 4.5.1.2 (Bandelt *et al.* 2001). The non-parametric bootstrap (henceforth referred to simply as the bootstrap) is a computer-based statistical technique that uses data resampling to estimate values of interest. The bootstrap sample is then analysed to infer a phylogenetic tree. The exact relationship of bootstrap values, Pboot, to posterior probability values, $P(\tau \mid D)$ is an open and important question in phylogenetic analysis. While the theory of each measure is largely independent, it has been posited that they should be equivalent. Therefore, a need exists for additional studies focusing on the behaviour and relationship of the bootstrap and posterior probability measures. Unfortunately, an analytical solution is not readily apparent for this study. The experiment used a paired design to compare the bootstrap values obtained using these two procedures for 1.000 simulated samples for a single point in the model space.

This tree was modified by manually adding six taxa (namely*: Circus approximans, Accipiter badius, Accipiter melanoleucus, Accipiter minullus, Elanus scriptus* and *Elanus axillaris*), based on a consensus tree of random phylogenies.

Results & Discussions

Although, the total number of vultures in captivity is slightly less than required to maintain current levels of heterozygosity, the difference in the number of breeding vultures necessary to maintain allelic diversity is much greater. The current captive breeding population constitutes only 27% of this number, and political and logistical barrier separating the captive breeding facilities potentially hamper exchange of individuals and the maintenance of genetic diversity even further.

In spite of these changes in food supply, the Cape Griffon *(Gyps coprotheres)* of southern Africa was the only member of the genus considered to be in danger of global extinction until the late 1990s. This species is believed to have been affected by multiple threats (BirdLife International 2007). It was then recognized that populations of vultures endemic to South Asia were declining rapidly across the Indian subcontinent for unknown reasons (Pain *et al.* 2008). To help mitigate this marked reduction in abundance, supplementary feeding stations (SFS; colloquially termed "vulture restaurants") have been created worldwide, often without consideration of the scientific evidence supporting the suitability of the practice (Cortes *et al.*

2016). In order to ensure that wild vulture populations recover and that a safe environment exists for captive-bred vultures to be released, the veterinary use of diclofenac and other similarly vulture-toxic NSAIDs needs to be eliminated. This is a huge challenge, but one that must be undertaken to prevent the extinction of these iconic birds (Mukherjee *et al.* 2014).

Far less information is available on the population trends of vultures in Nepal, as until relatively recently political instability in the country has prevented repeat surveys from being undertaken in lowland areas where resident Oriental White-backed and Slender-billed Vultures were formerly most abundant (Chaudhary *et al.* 2012). Globally, 61% of vulture species are threatened with extinction and are declining mainly due to anthropogenic pressures (Ogada *et al.* 2012). It is vital to understand threats to vultures in terms of land use and local human livelihoods. Previous research in Africa focused on the human dimensions of vulture conservation in commercial farming and protected areas (Pfeiffer *et al.* 2015).

This vulture population in Pakistan was experiencing rapid decline from diclofenac poisoning during the monitoring period, and so nest densities could have been lower than a potential maximum. Apart from differences in habitat (arid Nagarparkar, wetland dominated Keoladeo, coastal mangroves Sundarbans and forest plantation Changa Manga), the way spatial extent of the breeding areas in each study area may provide another explanation. Possible solutions of conservation, reintroducing in to the nature. Long-term effects of the problem of current technical bottlenecks that prevent better use of genomics to resolve conservation issues of vultures. Possibilities of using NSAID drugs in breeding and remove the harmful drugs from the food chain.

Result of this study suggest that this age-class is potentially still at risk in vulture population. Even with the goal of complete removal of diclofenac and other harmful non-steroidal anti-inflammatory drugs from the environment, it is possible that residual quantities of diclofenac remain in livestock carcasses and threat vultures. The establishment of a Vulture Safe Zone (VSZ) in the study area in 2012 saw the beginning of a new phase of environmental monitoring and conservation to address this issue. Across the approximately 8,000 km^2 VSZ, a range of activities such as livestock health camps, awareness-raising sessions in villages and consultations with veterinary dispensaries are all aimed at highlighting the risks to vultures from diclofenac and emphasizing the need to maintain the ban on its use in livestock. We have sequenced eleven samples for the identification of single nucleotide polymorphisms (SNP). There were two SNPs, and three heterozygous conditions were identified *(Table 1)*. T>G and G>A conversions were identified at positions 1 and

Table 1. SNPs, Heterozygous conditions and mutation types
1. táblázat SNP-k (egypontos nukleotid-polimorfizmusok), heterozigóta állapotok és mutáció típusok

Position	RefSeq	Changed position	Total samples	Transition/Transversion
1	T	G	V5	Transversion
2	G	A	V1, V3, V4, V5, V7, V10, V11	Transition
53	T/C	(Heter.)	V1, V2, V3, V4, V5, V7, V9, V10, V11	
110	T/C	(Heter.)	V1, V2, V3, V4, V5, V7, V9, V10, V11	
130	T/G	(Heter.)	V1, V2, V3, V4, V5, V7, V9, V10, V11	

2, respectively, as compared with the reference sequence (A/C). Two T/C heterozygous positions (53 and 110), and one T/G heterozygous position (130) were identified in all samples *(Table 1)*. However mtDNA is haploid, heteroplasmy (more than one mtDNA type in an individual) may occur. It is now thought that all individuals are heteroplasmic at some level – many above the limits of detection in DNA sequence analysis – thus heteroplasmy can be explanation for the occurrence of heterozygous positions in our sequences. All the other genetic information is given in *Table 1*.

Furthermore, an important next step in the monitoring of this colony is to determine breeding success. Comparing breeding success with pre-decline populations (and those that were suffering acute mortality from diclofenac poisoning could offer an indication of what levels of additive mortality exist for this population. Similarly, comparison of breeding productivity with the nearby colony of Long-billed Vultures will be important to see if the colonies are both (or neither) affected by similar rates of mortality which changes the genetic diversity of the vultures.

Finally, dispersal behavior of birds from this population must also be assessed. Oriental White-backed Vultures can range over vast distances, so it is not unlikely that birds may be dispersing across a wide area in the same way that birds may have arrived to the Nagarparkar

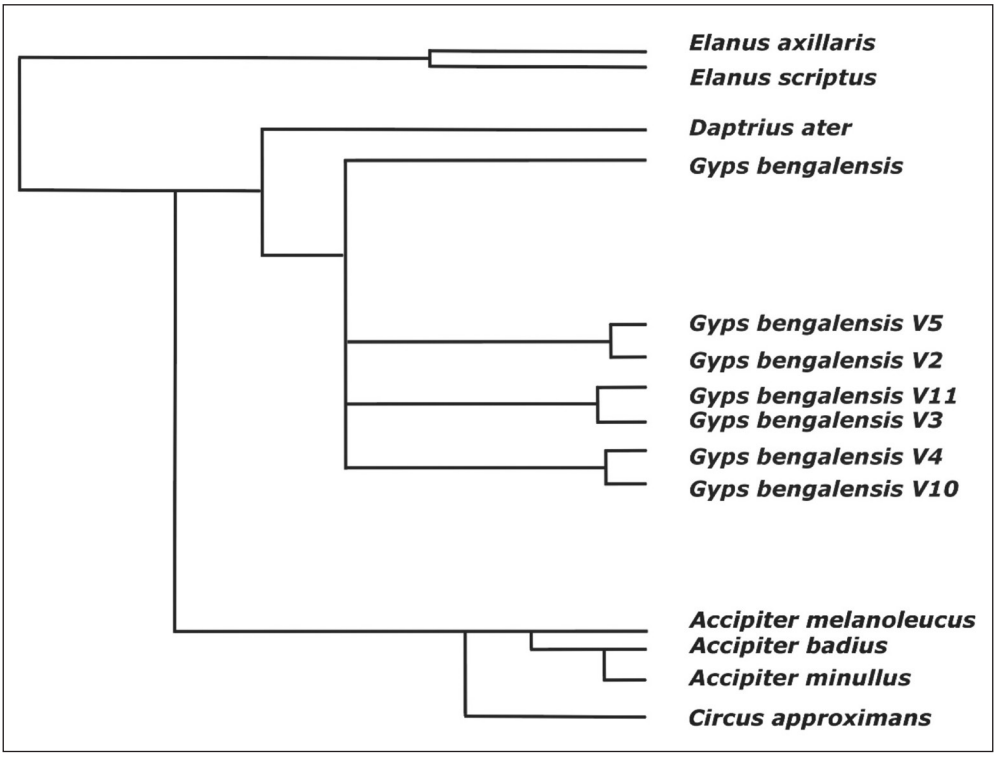

Figure 2. Phylogenetic analysis of samples of *Gyps bengalensis*. Other species on the tree represent raptorial birds

2. ábra A bengál keselyű minták filogenetikus analízise. A fán szereplő többi faj a ragadozómadarakat képviseli

colony from adjacent areas such as Gujarat in India. Looking at the results of molecular diversity and a phylogenetic analysis of Oriental White-backed Vultures, using a mitochondrial D-loop marker, no new species was identified. In samples V5 and V2, V11 and V3, as well as V4 and V10, share a common clade and have a common ancestor. Reference sequences, V9, V7 and V1, appeared to closely be related to each other. Sequence samples V5 and V6 of *Gyps bengalensis* are more closely related to *Gyps bengalensis* reference material *(Figure 2)*.

However, the long-term conservation value of a Vulture Safe Zone will be reduced if there are limited opportunities for vultures to nest in the spatial patterns that optimize the dynamics of their breeding colonies. Based on the results presented here in genetic analysis of mitochondrial D loop marker, and in addition to the removal of unsafe veterinary drugs, a key component of Vulture Safe Zone work should be the preservation of nest tree distributions that can support large colonies of clustered nests of Oriental White-backed Vultures.

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