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Ecological niche affects mitochondrial DNA diversity and variation in near-threatened Himalayan vulture (*Gyps himalayensis*)

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Abstract. *Ummee C, Sitdhibutr R, Lertwatcharasarakul P, Kasorndorkbua C. 2023. Ecological niche affects mitochondrial DNA diversity and variation in near-threatened Himalayan vulture* (Gyps himalayensis). *Biodiversitas 24: 3630-3640.* The impact from the use of diclofenac on the Indian subcontinent is the main reason why the Himalayan vulture *Gyps himalayensis* Hume, 1869 has near-threatened conservation status. In particular, it has ecological niches different from those of other vultures in the same genus; however, there has been no systematic study on genetic diversity. This study analyzed the genetic diversity of Himalayan vultures that had migrated to Thailand during the winter in conjunction with samples from a limited GenBank database. The results identified no evidence of Himalayan vulture genetic diversity loss after *Gyps* vultures were affected by diclofenac since the 1990s and the values were related to raptors with stable population status, which may be the result of ecological niche. Genetic differences or group divided were found in the mitochondrial DNA (mtDNA) Control Region (CR) and Cyt b+CR except in Cytochrome b (Cyt b). The group division based on the results of genetic distance between CR and Cyt b+CR shows that the genetic distance between groups of CR was 10-12 times greater than that of Cyt b (0.771±0.055-0.923±0.084 and 0.076±0.068, respectively) and the difference was also present when analyzed with the combined data set of Cyt b+CR (0.448±0.036). This is an important indicator for the possible study of population structure through phylogeography, because the Cyt b from other studies did not indicate any genetic differences between populations of Himalayan vulture and other *Gyps* vultures, which may update conservation proposals to be more accurate and effective.

Keywords: Control region, Cytochrome b, Cyt b, genetic differentiation, genetic diversity, Himalayan vulture *Gyps himalayensis*

INTRODUCTION

Residues in carcasses from the use of diclofenac (a non-steroidal anti-inflammatory drug used to treat mild-to-moderate pain in humans) have resulted in population declines of more than 90-95% of *Gyps* vultures on the Indian subcontinent, with many species having endangered conservation status and the Himalayan vulture (*Gyps himalayensis* Hume, 1869) being classified as near-threatened (BirdLife International 2022). Himalayan vultures are threatened by habitat loss, food shortage, and high-voltage power systems (Siddique and Khan 2016; Tulsi 2022). There is also evidence of adverse effects of diclofenac on this species (Das et al. 2011).

The Himalayan vulture has a different ecological niche to those of other *Gyps* species. The former inhabits mountainous areas and high land plateaus, unlike other *Gyps* species that frequent the lowlands, resulting in the difference in the distribution range of the *Gyps* species from Sichuan, China to Mongolia. However, the extent of the distribution range of *G. himalayensis* in many areas is still unclear (Thiollay 1994; Kasorndorkbua et al. 2008; Li and Kasorndorkbua 2008; Lu et al. 2009; Sherub et al. 2017; Kasorndorkbua et al. 2019; Kasorndorkbua et al. 2021; BirdLife International 2022).

Many raptor species have been shown to have low genetic diversity, such as haplotype or nucleotide diversity, with a decrease in the number of haplotypes or variation sites due to different evolutionary pressures, such as the Pleistocene climate change, the last ice age or the industrial revolution during the 19th and 20th centuries. Examples of this include the Cinereous vulture (*Aegypius monachus* Linnaeus, 1766) (Poulakakis et al. 2008; Ganbold et al. 2020), Red kite (*Milvus milvus* Linnaeus, 1758)) (Roques and Negro 2005), Hierofalcons (*Falco cherrug* J.E.Gray, 1834, *Falco biarmicus* Temminck, 1825, *Falco rusticolus* Linnaeus, 1758, and *Falco jugger* J.E.Gray, 1834) (Nittinger et al. 2007), and Griffon vulture (*Gyps fulvus* Hablizl, 1783) (Mereu et al. 2017). Other species have stable levels of genetic diversity, such as the White-tailed sea-eagle (*Haliaeetus albicilla* Linnaeus, 1758) (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2012) or a high level of diversity, such as Osprey (*Pandion haliaetus* Linnaeus, 1758) (Monti et al. 2015) and Bearded vulture (*Gypaetus barbatus* Linnaeus, 1758) (Godoy et al. 2004).

The decrease in population during the last glacial maximum (approximately 20,000 years ago) has resulted in low genetic diversity of the Himalayan vulture (Zou et al. 2021) and the Cytochrome b (Cyt b) analysis identified four haplotypes within a haplogroup with close evolutionary relationships (Arshad et al. 2009), due to the limited number of biological samples.

Mitochondrial DNA (mtDNA) has been used to study the genetic diversity of raptors extensively and continuously. Wink (2007) has proposed that mitochondrial DNA in animal organisms mutates faster than in nuclear genes, protein coding of mtDNA mutates 10-20 times faster than protein coding from the nucleus. Within mitochondrial DNA, the intron (non-coding) has a higher degree of mutation than the exon (coding) region. For example, the mutation of the Control Region (CR) (Intron) is about 4-6 times higher than the Cyt b (Exon) loci (Wink 2007).

Many raptors, including Himalayan vultures, make use of the specific geographical characteristics of Thailand in their migratory path. During migration, many vultures were found exhausted and suffered from food shortages, sometimes resulting in death. Due to the active vigilance and awareness of the local Thai network of villages, birdwatchers, national park rangers, many of the vultures were rescued and rehabilitated before release back to the wild. During every migratory season, up to 10-30 vultures may be found along its flyway corridor on the mountain ranges from North-Eastern, Western and Southern Thailand (Kasorndorkbua et al. 2019). This provides a good opportunity for Thailand to collect biological samples to study the genetic diversity of Himalayan vultures. Consequently, samples collected in Thailand through analysis of genetic diversity and genetic distance of CR, Cyt b, and the combined data set of Cyt b+CR from this study could provide important information for determining the effective conservation of the genetic diversity of this ecologically-specific and near-threatened species.

MATERIALS AND METHODS

Study area

Himalayan vultures migrated through Thailand along East Asian-Australasian Flyway. Many juvenile vultures were found weak and starved. During the winter from December 2010 to March 2021, a total of 14 juvenile Himalayan vultures were rescued from the provinces of Thailand, including Phang Nga, Phuket, Krabi, Suphan Buri, Bangkok, Chachoengsao, Ranong, and Songkhla as shown in Figure 1 and Table 1. Sampling and veterinary care of all rehabilitated Himalayan vultures were approved by Institutional Laboratory Animal Care and Use Committee, Kasetsart University, Thailand, with permit nos. ACKU64-VET-002 and ACKU64-VET-052. Specimens are being archived at Raptor Rehabilitation Unit, the Veterinary Teaching Hospital, Kasetsart University, Kamphaengsaen campus, Nakhon Pathom province, with identification codes as KU and R followed by numerical. After sampling and marking by ringing on the right tarsus, the vultures were rehabilitated and released back to nature by the Raptor Rehabilitation Unit, Kasetsart University Veterinary Teaching Hospital Kamphaengsaen Campus, Thailand.

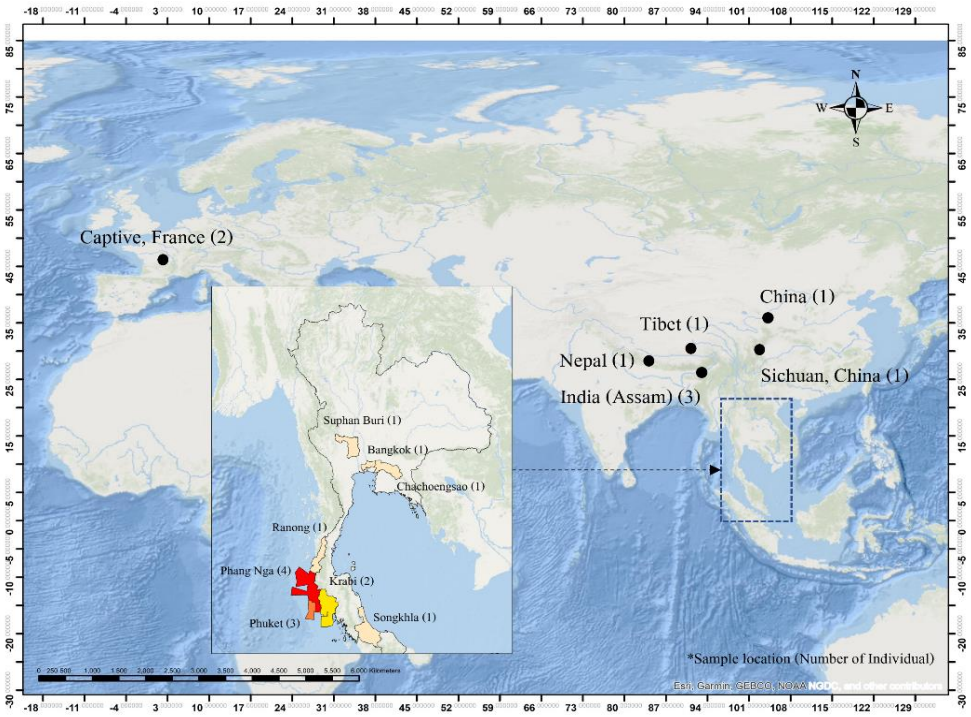

Figure 1. Origin locality of Himalayan vulture samplings, the intensity of the area color in Thailand is related to the increase of the number of individuals from each locality mapped using the QGIS program

Table 1. Sample information of Himalayan vultures being rescued in Thailand

ID	Sex	Age	Date of collection	Locality (Province)	Status	Sample type	GenBank acc. number	
							Cyt b	CR
KU61	Female	Juvenile	February 7, 2010	Songkhla	Dead	Muscle	OM362376	OM362390
R88	Male	Juvenile	January 2016	Phang Nga	Dead	Muscle	OM362377	OM362391
R89	Male	Juvenile	January 2016	Phang Nga	Dead	Muscle	OM362378	OM362392
KU155	Female	Juvenile	March 1, 2013	Phuket	Alive	Blood	OM362379	OM362393
KU233	Female	Juvenile	December 2013	Phang Nga	Alive	Blood	OM362380	OM362394
KU247	Female	Juvenile	December 14, 2014	Chachoengsao	Alive	Blood	OM362381	OM362395
KU301	Female	Juvenile	December 17, 2015	Suphan Buri	Alive	Blood	OM362382	OM362396
KU615	Female	Juvenile	March 22, 2018	Phuket	Alive	Blood	OM362383	OM362397
KU616	Male	Juvenile	March 22, 2018	Krabi	Alive	Blood	OM362384	OM362398
KU617	Male	Juvenile	March 22, 2018	Phang Nga	Dead	Muscle	OM362385	OM362399
KU618	Female	Juvenile	March 22, 2018	Phuket	Dead	Muscle	OM362386	OM362400
KU686	Male	Juvenile	January 7, 2019	Krabi	Alive	Blood	OM362387	OM362401
KU688	Female	Juvenile	January 20, 2019	Bangkok	Alive	Blood	OM362388	OM362402
KU852	Male	Juvenile	January 17, 2021	Ranong	Alive	Blood	OM362389	OM362403

Procedures

Sampling and DNA extraction, amplification, and sequencing

For each live bird, 0.5 mL of blood was collected in the metatarsal vein area using a blood collection tube containing Ethylenediaminetetraacetic Acid (EDTA) to prevent coagulation. For the deceased vultures, a 1.0 gram sample of pectoral muscles was taken and stored in a -20°C freezer.

DNA was extracted from the blood and tissue samples using a Blood Genomic DNA Extraction Mini Kit and Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech; Taiwan), respectively, with the extraction procedures following the company's recommendation. The DNA was amplified using Polymerase Chain Reaction (PCR) using the DreamTaq Green® PCR master mix enzyme (Thermo Scientific; Lithuania); the primer sets designed for this study were G_hima cyb F1: 5'CACCGGACTACTCAAAGCCTAC3'/G_hima cyb R2: 5'CTCAGTCTTTGGTTTACAAGACC3' in mtDNA Cyt b and G_hima Dloop F3: 5'GTTCTCTTCCCCTTAACTGG G3'/G_hima Dloop R4: 5'GGAGCTGGTGATAGAGG TTTGAG3' in CR with a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.; Hercules, CA, USA). Subsequently, the nucleotide sequencing was analyzed at BTSeq™ (Celemics, South Korea) using Illumina's next-generation sequencing technology.

The nucleotide sequence results were compared with the nucleotide sequences in the GenBank database to verify the quality and accuracy using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) and the BioEdit version 7.2.6.1 software (Hall 1999) and then deposited in the GenBank database with accession numbers OM362376-OM362389 (Cyt b) and OM362390-OM362403 (CR). Data management was performed through the 1,024 bp of Cyt b, 1,190 bp of CR, and 2,214 bp of Cyt b+CR. These were analyzed with reference sequences in the GenBank database at NCBI that consisted of six sequences, totaling nine individuals from different locations; from India (n=2), Nepal (n=1), and China (n=1) (INC), Assam, India (I; n=1), and Sichuan, China (SC; n=1) from the American Museum specimen from 1923 to 1956 (Johnson et al. 2006), from the captive in France (Zoo) (CF1 and CF2; n=2) (Arshad et al. 2009), and from Dangxiong County, Tibet Autonomous Region, China (T; n=1) (Jiang et al. 2019) (Figure 1; Table 2).

Data analysis

Multiple sequence alignment was performed based on the ClustalW algorithm (Thompson et al. 2002) in the MEGA-X program (Kumar et al. 2018), and the nucleotide substitution model was determined using the jModelTest 2.1.10 software (Darriba et al. 2012), with selection based on the Bayesian information criterion (Schwarz 1978).

Genetic diversity, consisting of haplotype diversity (hd) and nucleotide diversity (π) (Nei 1987) were analyzed using the DnaSP version 6.12.03 software (Rozas et al. 2017) and the Arlequin version 3.5.2.2 software (Excoffier and Lischer 2010). Haplotype diversity consisted of analysis of the division of haplotypes by finding variation sites and nucleotide substitutions, haplotype frequency, number of haplotypes (h), the variance of haplotype diversity, the standard deviation of haplotype diversity, and haplotype diversity. Nucleotide diversity was based on the average number of nucleotide differences (k), the number of polymorphic sites (S), the total number of mutations (Eta), and nucleotide diversity.

Genetic distances were analyzed based on the MEGA-X program (Kumar et al. 2018) using pairwise distance estimation. Nucleotide substitution was based on the Juke-Cantor model which included the gamma distribution (G) and the gamma parameter was 1.00. The data subset was based on the partial deletion method with a site coverage cutoff of 95% and nucleotide substitution used the Kimura 2-parameter (K2) model. The outgroup-based analysis from the GenBank database utilized *Gyps bengalensis* Gmelin, 1788 accession numbers KJ506806 (Gb1), KJ506807 (Gb2), and KJ506808 (Gb3) and *Gyps fulvus* accession numbers NC036050 (Gf1) and KX893247 (Gf2), with a total of 770 bp to compare the genetic distance within the Himalayan vulture with the distance between Himalayan vulture and the vultures in the same genus.

Table 2. Reference sequence information of Himalayan vultures from the GenBank database

GenBank Acc. number	ID	Location	Number of individuals	mtDNA	Reference
DQ908962	INC	India, Nepal, China	2 (India), 1 (Nepal), 1 (China)	Cyt b	Johnson et al. (2006)
DQ908963	I	Assam, India	1	Cyt b	Johnson et al. (2006)
DQ908964	SC	Sichuan, China	1	Cyt b	Johnson et al. (2006)
EU496455	CF1	Captive, France (Zoo)	1	Cyt b	Arshad et al. (2009)
EU496458	CF2	Captive, France (Zoo)	1	Cyt b	Arshad et al. (2009)
KY594709	T	Tibet Autonomous Region	1	Cyt b and CR	Jiang et al. (2019)

RESULTS AND DISCUSSION

Previous studies have shown that predators with increasing population trends and high genetic diversity, such as Osprey (Monti et al. 2015) and Bearded vulture (Godoy et al. 2004), the stable population trend and normal genetic diversity is the White-tailed sea-eagle (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2012), and several species show a decrease in population trend and low genetic diversity, including Red kite (Roques and Negro 2005), Cinereous vulture (Poulakakis et al. 2008), White-rumped vulture (*G. bengalensis*) (Johnson et al. 2008), Griffon vulture (Mereu et al. 2017), and Hierofalcons (Nittinger et al. 2007). The genetic diversity data of these raptors were used to compare with the genetic diversity of the Himalayan vulture with the following topics:

Dividing haplotypes

The nucleotide sequence from the Cyt b and CR divided the haplotype into seven haplotypes. In addition, the Cyt b+CR dataset could be split into 10 haplotypes. The vulture from the Tibet Autonomous Region, China (T) had a unique haplotype in all datasets. Based on Cyt b, there included seven variable sites, two parsimony informative, six nucleotide substitutions in transition, and two transversions (Table 3), whereas for CR, there included 15 variable sites, 10 parsimony informative, and 33 transitions and six transversions (Table 4), while Cyt b+CR included 20 variable sites, 11 parsimony informative, and 40 transitions and seven transversions (Table 5).

The Cyt b data indicated the number of variable sites of the Himalayan vulture was seven, which was much less than the 34 variable sites for the Osprey (Monti et al. 2015) and the 14 for the Cinereous vulture (Poulakakis et al. 2008). However, the analysis of the CR data showed that the Himalayan vulture had 15 variable sites, which was less than the 28 for the Bearded vulture (Godoy et al. 2004), but close to the 12-31 sites for the White-tailed sea-eagle (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2012). Furthermore, the number of variable sites in the CR data for the Himalayan vulture was higher than for the Red kite, which had 10 sites (Roques and Negro 2005), and the Griffon vulture in Europe, which had only two sites (Mereu et al. 2017). However, according to the analysis of CR, the Himalayan vulture did not have Indel (insertion-deletion mutations) similar as that of the Red kite (Roques and Negro 2005), but Bearded vulture, there are up to three Indels (Godoy et al. 2004) and White-tailed sea-eagles had up to 0-2 Indels (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2012).

From the Cyt b data, the Himalayan vulture had six transition nucleotide substitution sites, which was less than the 29 nucleotide substitution sites for the Cinereous vulture, with two transversion sites for the Himalayan vulture, while the Cinereous vulture had 13 such sites (Poulakakis et al. 2008). The CR dataset showed that the Himalayan vulture had 33 transitions, which was higher than the 15 transitions in the Red kite (Roques and Negro 2005), but lower than the 340 transitions in the Bearded vulture (Godoy et al. 2004) and the 60-82 transitions for the White-tailed sea-eagle (Hailer et al. 2007; Honnen et al. 2010). Similarly, there were six transversions in the Himalayan vulture CR, which was higher than for the Red kite which had no transversion sites, reflecting the short evolutionary period after the population decline during the 19th and 20th centuries (Roques and Negro 2005). In contrast, Bearded vulture had as many as 33 transversions (Godoy et al. 2004) and White-tailed sea-eagle had 2-13 transversions (Hailer et al. 2007; Honnen et al. 2010), reflecting their high genetic diversity and long evolutionary periods. This corresponded to three variants of nucleotide sequence sites: one in the Bearded vulture (Godoy et al. 2004) and one in the Central European White-tailed sea-eagle (Honnen et al. 2010); however, there were none for the Red kite (Roques and Negro 2005) or the White-tailed sea-eagle studied by Hailer et al. (2007) and the Himalayan vulture in this study. In addition, there were 10 parsimony information locations in the CR of the Himalayan vulture, which was lower than for the Bearded vulture that had a maximum of 21 sites (Godoy et al. 2004). While the Cyt b of the Himalayan vulture showed only 2 parsimony informative sites (Table 6).

Haplotype diversity

The Cyt b position had a haplotype diversity of 0.68950 (variance 0.01108, SD 0.10500). The CR position had haplotype diversity of 0.78100 (variance 0.01031, SD 0.10200). The Cyt b+CR dataset had a haplotype diversity of 0.89500 (variance 0.00496, SD 0.07000). Based on the Cyt b data, Himalayan vultures in this study had seven haplotypes (n=20), which was more numerous than the four haplotypes reported by Arshad et al. (2009) using Cyt b of 1,026 bp (n=8). While there were fewer than Ospreys with 19 haplotypes than reported by Monti et al. (2015), there was the same number of Cinereous vultures (n=48) as for Poulakakis et al. (2008). The analysis of the CR loci indicated that the Himalayan vulture had seven haplotypes,

which was less than that for Bearded vulture (up to 50 haplotypes were reported in Godoy et al. 2004), and White-tailed sea-eagle (13-38 haplotypes were reported in Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2012). The number of haplotypes in Himalayan vulture in this study is comparable to White-rumped vulture experiencing depopulation in the Indian subcontinent, with haplotype numbers in the range 7-13 (Johnson et al. 2008), and previous declining populations of Red kite, with 10 haplotypes reported (Roques and Negro 2005) (Table 7).

Table 3. Variable site and haplotype frequency of Himalayan vulture mtDNA Cyt b

Haplotype	Site no.							Sample	Count
	25	596	652	655	828	930	952		
HCb1	G	G	C	T	C	A	C	INC, CF1, KU61, R89, KU233, KU301, KU615, KU616, KU618, KU688, KU852	11
HCb2	.	A	.	.	.	.	.	CF2	1
HCb3	.	.	T	.	.	.	.	I, R88, KU155	3
HCb4	.	.	.	.	T	.	G	SC, KU247	2
HCb5	.	A	.	.	.	C	.	T	1
HCb6	.	.	.	C	.	.	.	KU617	1
HCb7	A	.	.	.	.	.	.	KU686	1
Total									20

Note: CF1: Captive, France (Zoo) 1, CF2: Captive, France (Zoo) 2, I: Assam, India, INC: India, Nepal, and China, KU and R: Thailand, SC: Sichuan, China, T: Tibet Autonomous Region, China

Table 4. Variable site and haplotype frequency of Himalayan vulture mtDNA CR

Haplotype	Site no.															Sample	Count
	57	58	71	72	147	251	258	261	326	332	338	342	346	397	460		
HCr1	A	C	T	A	G	C	G	G	A	G	A	C	T	C	A	KU61, R89, KU247, KU616, KU618, KU686, KU688	7
HCr2	G	T	.	.	A	A	A	A	G	.	G	T	.	.	.	R88	1
HCr3	G	T	.	.	A	A	A	A	G	.	.	T	.	.	.	KU155	1
HCr4	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	KU233, KU617	2
HCr5	.	.	.	.	.	.	.		.	.	.	.	.	.	G	KU301	1
HCr6	G	T	.	.	A	A	A	A	G	A	.	T	.	.	.	KU615, KU852	2
HCr7	G	T	C	C	A	A	A	A	G	.	.	T	.	G	.	T	1
Total																	15

Note: KU and R: Thailand, T: Tibet Autonomous Region, China

Table 5. Variable site and haplotype frequency of Himalayan vulture mtDNA Cyt b+CR

Haplotype	Site no.																					Sample	Count
	25	596	652	655	930	1081	1082	1095	1096	1171	1275	1282	1285	1350	1356	1362	1366	1370	1421	1485			
HCbCr1	G	G	C	T	A	A	C	T	A	G	C	G	G	A	G	A	C	T	C	A		KU61, R89, KU616, KU618, KU688	5
HCbCr2	.	A	.	.	.	G	T	.	.	A	A	A	A	G	.	G	T	.	.	.		R88	1
HCbCr3	.	A	.	.	.	G	T	.	.	A	A	A	A	G	.	.	T	.	.	.		KU155	1
HCbCr4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.		KU233	1
HCbCr5	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		KU247	1
HCbCr6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G		KU301	1
HCbCr7	.	.	.	.	.	G	T	.	.	A	A	A	A	G	A	.	T	.	.	.		KU615, KU852	2
HCbCr8	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.		KU617	1
HCbCr9	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		KU686	1
HCbCr10	.	A	.	.	C	G	T	C	C	A	A	A	A	G	.	.	T	.	G	.		T	1
Total																							15

Note: KU and R: Thailand; T: Tibet Autonomous Region, China

Table 6. Comparison of variable sites of the Himalayan vulture and other raptors

Species	Marker	bp	Variable site	Indel	Transition	Transversion	TVNSS	PI	Reference
Cinereous vulture	COI	738	3	0	1	2	0	-	Ganbold et al. (2020)
Cinereous vulture	Cyt b	311	14	-	29	13	-	-	Poulakakis et al. (2008)
Osprey	Cyt b	661	34	-	-	-	-	-	Monti et al. (2015)
Himalayan vulture	Cyt b	1,024	7	0	6	2	0	2	This study
Bearded vulture	CR	228	28	3	340	33	1	21	Godoy et al. (2004)
Red kite	CR	357	10	0	15	0	0	-	Roques and Negro (2005)
White-tailed sea-eagle	CR	500	12	0	60	2	0	-	Hailer et al. (2007)
Hierofalcons	CR	412	97	-	-	-	-	-	Nittinger et al. (2007)
White-tailed sea-eagle	CR	499	21	1	82	13	1	-	Honnen et al. (2010)
White-tailed sea-eagle	CR	499	31	2	-	-	-	-	Langguth et al. (2012)
Griffon vulture	CR	447	2	-	-	-	-	-	Mereu et al. (2017)
Himalayan vulture	CR	1,190	15	0	33	6	0	10	This study

Note: TVNSS: Three Variants of Nucleotide Sequence Site, PI: Parsimony Informative

Table 7. Comparison of haplotype diversity of the Himalayan vulture and other raptors

Species	Marker	bp	n	Number of haplotypes	Haplotype diversity	Reference
Cinereous vulture	COI	738	39	4	0.27900±0.00790	Ganbold et al. (2020)
Cinereous vulture	Cyt b	311	48	7	-	Poulakakis et al. (2008)
White-rumped vulture	Cyt b	1,026	38	12	-	Arshad et al. (2009)
Himalayan vulture	Cyt b	1,026	8	4	-	Arshad et al. (2009)
Griffon vulture	Cyt b	1,026	60	12	-	Arshad et al. (2009)
Rüppell's vulture	Cyt b	1,026	6	5	-	Arshad et al. (2009)
Slender-billed vulture	Cyt b	1,026	13	2	-	Arshad et al. (2009)
Indian vulture	Cyt b	1,026	51	5	-	Arshad et al. (2009)
Cape vulture	Cyt b	1,026	7	4	-	Arshad et al. (2009)
White-backed vulture	Cyt b	1,026	77	14	-	Arshad et al. (2009)
Osprey	Cyt b	661	146	19	0.79500	Monti et al. (2015)
Himalayan vulture	Cyt b	1,024	20	7	0.68950	*This study*
Bearded vulture	CR	228	172	50	0.93200±0.01200	Godoy et al. (2004)
Red kite	CR	357	105	10	0.61000	Roques and Negro (2005)
White-tailed sea-eagle	CR	500	228	13	0.74600	Hailer et al. (2007)
Hierofalcons	CR	412	244	5-57	0.80200	Nittinger et al. (2007)
White-rumped vulture	CR	396	19-26	7-13	0.76000-0.84600	Johnson et al. (2008)
White-tailed sea-eagle	CR	499	166	23	0.76400	Honnen et al. (2010)
White-tailed sea-eagle	CR	499	420	38	0.79700	Langguth et al. (2012)
Griffon vulture	CR	447	66	3	0.57400±0.03300	Mereu et al. (2017)
Himalayan vulture	CR	1,190	15	7	0.78100	*This study*

The Cyt b+CR dataset of Himalayan vulture in this study had the highest haplotype diversity, followed by CR and Cyt b loci, respectively. Based on Cyt b, there was no evidence of genetic diversity loss in the Himalayan vulture. The Cyt b showed more haplotypes from museum specimens collected between 1923 and 1956 that led to multiple unique haplotypes in the samples collected from 2008 to 2021. These periods were before and after the decrease in the number of *Gyps* vultures in the Indian subcontinent (around 1990s), which was probably affected by diclofenac. The haplotype diversity in the Cyt b for the Himalayan vulture was 0.68950, which was less than the 0.79500 for the Osprey (Monti et al. 2015). The CR haplotype diversity in the Bearded vulture was 0.93200 (Godoy et al. 2004), which was higher for the Himalayan vulture (0.78100). The Himalayan vulture had a similar haplotype diversity value to the White-tailed sea-eagle, in the range 0.74600-0.79700 (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2012) and to the White-rumped vulture (whose population has declined to an endangered state but they can maintain levels of genetic diversity due to the small population size and short evolutionary timescale) with values in the range 0.76000-0.84600 (Johnson et al. 2008). But the Himalayan vulture had a higher haplotype diversity value than the Red kite whose population had declined during the 19th and 20th centuries to only 0.61000 (Roques and Negro 2005) (Table 7).

Nucleotide diversity

Nucleotide diversity (π) in Cyt b was 0.00100, the average number of nucleotide differences (k) was 1.02600, and the number of polymorphic sites (S) and the total number of mutations (Eta) was seven positions. Based on CR, the nucleotide diversity was 0.00420, the average

number of nucleotide differences was 4.97100, and the number of polymorphic sites and the total number of mutations was 15 positions. The combination of the Cyt b+CR dataset produced nucleotide diversity of 0.00265, the average number of nucleotide differences was 5.84800, and the numbers of polymorphic sites and the total number of mutations was 20. The Himalayan vulture had the highest nucleotide diversity with CR (π=0.00420), followed by with Cyt b+CR (π=0.00265) and Cyt b (π=0.00100), respectively. The Himalayan vulture had less nucleotide diversity in Cyt b than the Osprey (π=0.00100 and 0.01060, respectively), which is a successful raptor with increasing populations and distributed throughout the world (Monti et al. 2015). In the CR dataset, the nucleotide diversity of Himalayan vulture was lower (π=0.00420), compared with that of Bearded vulture (π=0.02900) (Godoy et al. 2004) and White-tailed sea-eagle (π=0.00680-0.00718) (Hailer et al. 2007; Honnen et al. 2010; Langguth et al. 2012). On the other hand, the Himalayan vulture had a higher nucleotide diversity than the raptors whose populations were affected by the 19th and 20th century industrial revolution including Red kite (π=0.00320±0.00200 in Roques and Negro 2005), and Hierofalcons (π=0.00300) (Nittinger et al. 2007) and European Griffon vulture (0.00125-0.00179) (Mereu et al. 2017) (Table 8).

Genetic distance

The nucleotide sequence of Cyt b of Himalayan vulture had a percentage range of uncorrected p-distance of 0.000-0.393%, the largest distance being for vultures from the Tibet Autonomous Region (T) and the vulture from France zoo (CF2) (Table 9). The analysis of the CR location indicated the distance was in the range 0.000-1.049% (Tables 10 and 11). In the Cyt b+CR dataset, the genetic distance range was 0.000-0.686% (Table 12). Genetic distances between groups of mtDNA CR were 0.771±0.055-0.923±0.084% while Cyt b was 0.076±0.068% as shown in the gray area of Tables 9-11, while the Cyt b+CR was 0.448±0.036% (Table 12). The groups based on CR and Cyt b+CR results, including the group of KU61, R89, KU233, KU247, KU301, KU616, KU617, KU618, KU686, and KU688 and the group of R88, KU155, KU615, and KU852.

The average genetic distance based on CR between the Himalayan vulture and *G. bengalensis* was 2.613% (2.253-2.794%) and the distance with *G. fulvus* was 1.850% (1.708-2.109%). The average genetic distance within Himalayan vultures from the groups composed of KU233, KU247, KU301, KU616, KU617, KU618, KU686, and KU688 were different from the groups composed of R88, KU155, KU615, KU852, and T was 0.929% (0.784-1.049%), as shown in Table 11.

Analysis of all samples showed that the Himalayan vulture CR data had a greater genetic distance than the Cyt b+CR and Cyt b datasets, respectively. Based on analysis of the CR loci, *Gyps* vultures had a genetic distance between species in the range 1.300-4.000% (Johnson et al. 2006). In the current study, Himalayan vulture CR has 10-12 times more genetic distance between groups than Cyt b (0.771±0.055-0.923±0.084% and 0.076%, respectively) (Figure 2). In the CR, the average genetic distance between groups of *G. himalayensis* was 0.923%, which is more than half the genetic distance to *G. fulvus* (1.850%) and the genetic distance between *G. himalayensis* and *G. fulvus* and the highest genetic distance between the groups of *G. himalayensis* were similar, 1.708% and 1.049%, respectively. The genetic distance between the groups of Himalayan vultures was similar to the genetic distance between the population of the white-tailed sea-eagle, 0.771-0.923% and 0.700%, respectively (Hailer et al. 2007) and the white-tailed sea eagle in central Europe, 1.049% and 1.200%-1.400% (Honnen et al. 2010), respectively. While Cyt b has a smaller genetic distance than Osprey (Monti et al. 2015) (Table 13). These findings show that CR has greater genetic variation than Cyt b and was even greater when analyzed with Cyt b+CR used for reducing data bias (Bull et al. 1993; Cunningham 1997; Wiens 1998). This CR locus may be a marker for confirming the isolation of Himalayan vulture populations based on their distribution range.

Table 8. Comparison of nucleotide diversity of the Himalayan vulture and other raptors

Species	Marker	bp	n	Nucleotide diversity	Reference
Osprey	Cyt b	661	146	0.01060	Monti et al. (2015)
Himalayan vulture	Cyt b	1,024	20	0.00100	*This study*
Bearded vulture	CR	228	172	0.02900±0.01500	Godoy et al. (2004)
Red kite	CR	357	105	0.00320±0.00200	Roques and Negro (2005)
White-tailed sea-eagle	CR	500	228	0.00680±0.00010	Hailer et al. (2007)
Hierofalcons	CR	412	244	0.00300	Nittinger et al. (2007)
White-tailed sea-eagle	CR	499	123	0.00698	Honnen et al. (2010)
White-tailed sea-eagle	CR	499	102	0.00718	Langguth et al. (2012)
Griffon vulture	CR	447	66	0.00125-0.00179	Mereu et al. (2017)
Himalayan vulture	CR	1,190	15	0.00420	*This study*

Table 9. Percentage of Himalayan vulture mtDNA Cyt b genetic distance (1,024 bp)

	SC	I	INC	CF1	CF2	KU61	R89	KU233	KU247	KU301	KU616	KU617	KU618	KU686	KU688	R88	KU155	KU615	KU852
SC																			
I	0.196																		
INC	0.098	0.098																	
CF1	0.098	0.098	0.000																
CF2	0.294	0.294	0.196	0.196															
KU61	0.098	0.098	0.000	0.000	0.196														
R89	0.098	0.098	0.000	0.000	0.196	0.000													
KU233	0.098	0.098	0.000	0.000	0.196	0.000	0.000												
KU247	0.000	0.196	0.098	0.098	0.294	0.098	0.098	0.098											
KU301	0.098	0.098	0.000	0.000	0.196	0.000	0.000	0.000	0.098										
KU616	0.098	0.098	0.000	0.000	0.196	0.000	0.000	0.000	0.098	0.000									
KU617	0.196	0.196	0.098	0.098	0.294	0.098	0.098	0.098	0.196	0.098	0.098								
KU618	0.098	0.098	0.000	0.000	0.196	0.000	0.000	0.000	0.098	0.000	0.000	0.098							
KU686	0.196	0.196	0.098	0.098	0.294	0.098	0.098	0.098	0.196	0.098	0.098	0.196	0.098						
KU688	0.098	0.098	0.000	0.000	0.196	0.000	0.000	0.000	0.098	0.000	0.000	0.098	0.000	0.098					
R88	0.196	0.000	0.098	0.098	0.294	0.098	0.098	0.098	0.196	0.098	0.098	0.196	0.098	0.196	0.098				
KU155	0.196	0.000	0.098	0.098	0.294	0.098	0.000	0.098	0.196	0.098	0.098	0.196	0.098	0.196	0.098	0.000			
KU615	0.098	0.098	0.000	0.000	0.196	0.000	0.000	0.000	0.098	0.000	0.000	0.098	0.000	0.098	0.000	0.098	0.098		
KU852	0.098	0.098	0.000	0.000	0.196	0.000	0.000	0.000	0.098	0.000	0.000	0.098	0.000	0.098	0.000	0.098	0.098	0.000	
T	0.295	0.098	0.196	0.196	0.393	0.196	0.196	0.196	0.295	0.196	0.196	0.295	0.196	0.295	0.196	0.098	0.098	0.196	0.196

Note: CF1: Captive, France (Zoo) 1, CF2: Captive, France (Zoo) 2, I: Assam, India, INC: India, Nepal, and China, KU and R: Thailand, SC: Sichuan, China, T: Tibet Autonomous Region, China, Grey area: Genetic distance between group

Table 10. Percentage of Himalayan vulture mtDNA CR genetic distance (1,190 bp)

	KU61	R89	KU233	KU247	KU301	KU616	KU617	KU618	KU686	KU688	R88	KU155	KU615	KU852	T
KU61															
R89	0.000														
KU233	0.084	0.084													
KU247	0.000	0.000	0.084												
KU301	0.084	0.084	0.169	0.084											
KU616	0.000	0.000	0.084	0.000	0.084										
KU617	0.084	0.084	0.000	0.084	0.169	0.084									
KU618	0.000	0.000	0.084	0.000	0.084	0.000	0.084								
KU686	0.000	0.000	0.084	0.000	0.084	0.000	0.084	0.000							
KU688	0.000	0.000	0.084	0.000	0.084	0.000	0.084	0.000	0.000						
R88	0.767	0.767	0.853	0.767	0.853	0.767	0.853	0.767	0.767	0.767					
KU155	0.681	0.681	0.767	0.681	0.767	0.681	0.767	0.681	0.681	0.681	0.084				
KU615	0.767	0.767	0.853	0.767	0.853	0.767	0.853	0.767	0.767	0.767	0.169	0.084			
KU852	0.767	0.767	0.853	0.767	0.853	0.767	0.853	0.767	0.767	0.767	0.169	0.084	0.000		
T	0.940	0.940	1.027	0.940	1.027	0.940	1.027	0.940	0.940	0.940	0.339	0.254	0.339	0.339	

Note: KU and R: Thailand, T: Tibet Autonomous Region, China, Grey area: Genetic distance between group

Table 11. Percentage of Himalayan vulture mtDNA CR genetic distance (770 bp)

	Gb1	Gb2	Gb3	Gf1	Gf2	KU61	R89	KU233	KU247	KU301	KU616	KU617	KU618	KU686	KU688	R88	KU155	KU615	KU852	T
Gb1																				
Gb2	0.000																			
Gb3	0.000	0.000																		
Gf1	4.018	4.018	4.018																	
Gf2	4.018	4.018	4.018	0.000																
KU61	2.658	2.658	2.658	1.708	1.708															
R89	2.658	2.658	2.658	1.708	1.708	0.000														
KU233	2.794	2.794	2.794	1.841	1.841	0.130	0.130													
KU247	2.658	2.658	2.658	1.708	1.708	0.000	0.000	0.130												
KU301	2.794	2.794	2.794	1.841	1.841	0.130	0.130	0.260	0.130											
KU616	2.658	2.658	2.658	1.708	1.708	0.000	0.000	0.130	0.000	0.130										
KU617	2.794	2.794	2.794	1.841	1.841	0.130	0.130	0.000	0.130	0.260	0.130									
KU618	2.658	2.658	2.658	1.708	1.708	0.000	0.000	0.130	0.000	0.130	0.000	0.130								
KU686	2.658	2.658	2.658	1.708	1.708	0.000	0.000	0.130	0.000	0.130	0.000	0.130	0.000							
KU688	2.658	2.658	2.658	1.708	1.708	0.000	0.000	0.130	0.000	0.130	0.000	0.130	0.000	0.000						
R88	2.253	2.253	2.253	1.975	1.975	0.916	0.916	1.049	0.916	1.049	0.916	1.049	0.916	0.916	0.916					
KU155	2.388	2.388	2.388	1.975	1.975	0.784	0.784	0.916	0.784	0.916	0.784	0.916	0.784	0.784	0.784	0.130				
KU615	2.524	2.524	2.524	2.109	2.109	0.916	0.916	1.049	0.916	1.049	0.916	1.049	0.916	0.916	0.916	0.260	0.130			
KU852	2.524	2.524	2.524	2.109	2.109	0.916	0.916	1.049	0.916	1.049	0.916	1.049	0.916	0.916	0.916	0.260	0.130	0.000		
T	2.522	2.522	2.522	2.108	2.108	0.916	0.916	1.048	0.916	1.048	0.916	1.048	0.916	0.916	0.916	0.260	0.130	0.260	0.260	

Note: Gb: *Gyps bengalensis*, Gf: *Gyps fulvus*, KU and R: Thailand, T: Tibet Autonomous Region, China, Grey area: Genetic distance between group

Table 12. Percentage of Himalayan vulture mtDNA Cyt b+CR genetic distance (2,214 bp)

	KU61	R89	KU233	KU247	KU301	KU616	KU617	KU618	KU686	KU688	R88	KU155	KU615	KU852	T
KU61															
R89	0.000														
KU233	0.045	0.045													
KU247	0.045	0.045	0.091												
KU301	0.045	0.045	0.091	0.091											
KU616	0.000	0.000	0.045	0.045	0.045										
KU617	0.091	0.091	0.045	0.136	0.136	0.091									
KU618	0.000	0.000	0.045	0.045	0.045	0.000	0.091								
KU686	0.045	0.045	0.091	0.091	0.091	0.045	0.136	0.045							
KU688	0.000	0.000	0.045	0.045	0.045	0.000	0.091	0.000	0.045						
R88	0.455	0.455	0.501	0.501	0.501	0.455	0.547	0.455	0.501	0.455					
KU155	0.410	0.410	0.455	0.455	0.455	0.410	0.501	0.410	0.455	0.410	0.045				
KU615	0.410	0.410	0.455	0.455	0.455	0.410	0.501	0.410	0.455	0.410	0.136	0.091			
KU852	0.410	0.410	0.455	0.455	0.455	0.410	0.501	0.410	0.455	0.410	0.136	0.091	0.000		
T	0.594	0.594	0.640	0.640	0.640	0.594	0.686	0.594	0.640	0.594	0.227	0.182	0.273	0.273	

Note: KU and R: Thailand, T: Tibet Autonomous Region, China, Grey area: Genetic distance between group

Table 13. Comparison of genetic distance of the Himalayan vulture and other raptors

Species	Marker	bp	n	Genetic distance	Reference
Osprey	Cyt b	661	146	0.100-0.200% (Within population) and 1.200% (1.500-2.600%) (Between populations)	Monti et al. (2015)
Himalayan vulture	Cyt b	1,024	20	0.000-0.393% (Within group) and 0.076±0.068 % (Between group)	*This study*
Genus *Gyps*	CR	400	20	1.300-4.000% (Between species)	Johnson et al. (2006)
White-tailed sea-eagle	CR	500	228	0.700% (Between populations)	Hailer et al. (2007)
White-tailed sea-eagle	CR	499	123	1.200%-1.400% (Between populations)	Honnen et al. (2010)
Himalayan vulture	CR	770-1,190	14	0.000-0.260% (Within group) and 0.681-1.049% (Between group)	*This study*

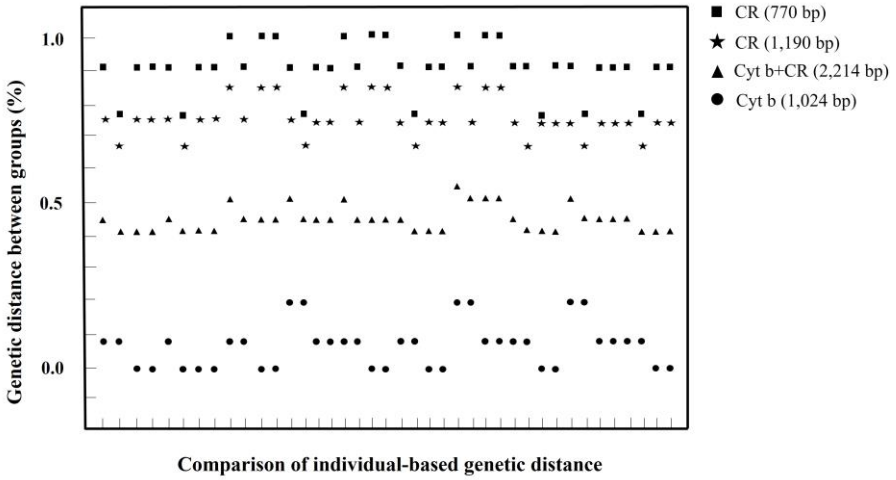

Figure 2. Comparison diagram of Himalayan vulture genetic distance between groups with different loci

The effect of dividing haplotype, genetic diversity, and genetic distance may depend on the number of samples in the current study (15-20 individuals), as opposed to studies in other comparative raptors where sample numbers were in the range of 66-228 individuals. Nonetheless, the apparent genetic differentiation of the CR in the current study was more variable than for the Cyt b data in another study that suggested there was no geographically divided population structure for *Gyps* vultures (Arshad et al. 2009). Furthermore, the unique haplotypes are found in many areas, such as the Tibetan Plateau. It indicates the need to conserve genetic diversity according to the genetic differences of each area without addressing the conservation of the near-threatened species as a species-level overview (Avise et al. 1987).

In conclusion, there was no evidence of loss of genetic diversity for Himalayan vultures based on the sample in the current study from Thailand and the limited databases available from elsewhere from using several methods (dividing haplotypes, haplotype diversity, nucleotide diversity, and genetic distance of mtDNA Cyt b, CR, and Cyt b+CR). The Himalayan vulture tended to have genetic diversity values similar to those of stable-state raptors. This may be associated with an ecological niche which is less exposed to diclofenac on the Indian subcontinent than that for other *Gyps* species. This may be because the area is just a non-breeding area of the Himalayan vulture (winter visitor). They return to build nests and lay eggs in the highlands, while other *Gyps* vultures are resident birds that live on the Indian subcontinent throughout the year. However, the genetic differences indicating greater variance in CR than Cyt b and also confirmed by Cyt b+CR provided important data that can be used to study the population structure through analyses of phylogeography to quantify population genetic variation in each area of the distribution range. In particular, the ecological niche of the Himalayan vulture in relation to mountain ranges and plateaus may influence population structure. This will be crucial information for planning genetic diversity conservation that is different from what has been proposed in earlier studies.

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