To sequence:
Cyt b and ND2
       Terathopius (study skin)
       Spilornis cheela (study skin)
       Spilornis holospilus (study skin)
ND2
       Gypaetus barbatus (study skin)
       Neophron percnopteus (study skin)

       Sarcogyps (study skin)
       Trigonoceps (study skin)
       Torgos tracheliotus (1/2 done)
       Necrosyrtes monachus (#43, no ND2)
       Circaetus gallicus (Mindell tissue)
       Accipiter (maybe do two, Mindell tissues)
       Circus (do cyt b also? Mindell tissue)
       Pernis apivorus (no ND2, #17)
       Gypohierax angolensis ((#42, no ND2)

Taxa to include that are already sequenced
       Buteo (2),ACCIPITER?, milvus migrans, haliaeetus (2), Aegypius, Harpagus aquila (2),
Gyps (2), Elanus?, and outgroups (2; fape and one other)—see what happens to new guinea harpy
(recheck sequence for nd2 and cyt b)

**Polyphyly of the Old World Vultures and phylogenetic placement of *Gypohierax angolensis*
(Aves: Accipitridae) inferred from mitochondrial DNA**

Introduction

The palmnut vulture (*Gypohierax angolensis*) is a monotypic species found along the coast, estuaries and rivers of sub-Saharan Africa in areas where oil and raffia palms also occur. This vulture is relatively common but not abundant in Mozambique and rare in northern Zululand, Botswana and Zimbabwe; it is considered South Africa's rarest breeding bird. The diet of *Gypohierax* largely consists of palm fruits, but also includes many fish, crabs, snails and other small animals, as well as other birds. (Ginn et al., 1989)

An alternative name of *Gypohierax* is the vulturine fish eagle (Ginn et al., 1989), reflecting its many similarities, both dietary and morphological (especially talon structure), to the sea and fish eagles (genus *Haliaeetus*). The phylogenetic placement and evolutionary history of *Gypohierax* is unclear. Brown and Amadon (1968) included *Gypohierax* as a member of a monophyletic clade of Old world vultures, but also suggested a sister relationship between the sea eagles and *Gypohierax*. Jollie (1977), Suschkin (1899), and Brown and Amadon (1968) note that *Gypohierax* resembles the Egyptian vulture (*Neophron)* and may be a member of a monophyletic clade of all Old World vultures (including *Neophron)*. Holdaway (1994), in a phylogenetic analysis of osteological characters, finds support for a monophyletic clade of vultures where the palmnut vulture is the earliest diverging vulture.

Several phylogenetic analyses based on mitochondrial DNA and morphological traits support paraphyly of the Old World vultures (Seibold and Helbig, 1995; Mundy et al., 1992). Two clades are identified: the *Gyps-Aegypius-Necrosyrtes* clade includes the genera of its name as well as *Torgos*, *Trigonoceps*, and *Sarcogyps*, and is sister to the Snake eagles; and, the *Gypaetus-Neophron* clade, is sister to *Pernis apivorus*, including only the two genera for which it is named. *Gypohierax* is not included in these analyses, but Seibold and Helbig propose that it

most likely represents a third independent evolutionary line separate from the other Old World vulture clades.

In summary, it has been proposed that Gypohierax (1) forms a clade independent from other vulture lineages, (2) is sister to the sea eagles (*Haliaeetus*), (3) is a member of a monophyletic clade of Old World vultures, and (4) is most closely related to the Egyptian vulture (*Neophron*). The Old World vultures have been proposed to be (1) a monophyletic clade, (2) two separate distinct monophyletic clades (overall polyphyly), and (3) three separate distinct monophyletic clades (overall polyphyly).

Methods

DNA was extracted from tissue and feather samples using the Qiagen DNEasy Extraction kit. An addition of dithiothreitol was used for samples from feathers. Polymerase chain reaction (PCR) was carried out using two primers pairs for cytochrome B (CytB; Sorenson et al. 1999). PCR and sequencing was done following protocols in Mindell et al. (1997). Additional CytB sequences for analyses were obtained from GenBank (*Aegypius monachus,* gi 1050567; *Trigonoceps occipitalis,* gi 1050710*; Sarcogyps calvus,* gi 1050699*, Gypaetus barbatus,* gi 1050621; and *Neophron percnopterus*, gi 1050666).

Conceptually translated protein sequences were aligned by eye and this alignment was imposed on the nucleotides. Phylogenetic analyses were conducted using parsimony and maximum likelihood (ML) criteria for the dataset. For parsimony analysis, a bootstrap analysis was done using Winclada software (Nixon, 2002), 1000 replications with 100 search reps and ten starting trees per rep. A Bayesian inference (BI) approach, which is related to ML analyses, (Mau et al., 1999; Yang and Rannala) was performed with Metropolis-coupled Markov chain Monte Carlo, or $(MC)^3$, to approximate the posterior probabilities (PP) of the trees in MrBayes 2.1 (Huelsenbeck and Ronquist). Bayesian inference has advantages over other methods of phylogenetic inference in interpretation of results, consistency (Wilcox et al.) and computational speed (Large and Simon); however, as always, reliability of the results depends on

appropriateness of the model, and some simulations have demonstrated artifactually high PP support values (Suzuki et al., 2002). Base frequencies and gamma distribution (with eight rate categories) were estimated each run. A general time reversible model was used, which includes six substitution types. The search was run twice, starting from random trees with four simultaneous Markov chains, sampling every 50 generations for 1,000,000 generations. The proportion of searches in which any given node (set of relationships) is found during the chain is an approximation of its PP, and provides an indication of support for that node based on the dataset.

Results

The sequence data set of 1017 nucleotides from 27 species aligned with no indels or stop codons. Of the 1017 nucleotide positions, 458 were variable and 339 were parsimony informative.

Each amino acid is coded for by up to six different codons (synonymous codons). The relative frequency of each synonymous codon should be equal to that of the its related synonymous codons if none of the codons are favored by selection or mutation. However, many organisms have been found to have biases in synonymous codon usage (Sharp et al., 1988). Codon bias was also found in this data set of Accipitridae for most amino acids (table 1). The largest biases were found in valine (V) and serine (S), where one codon was at least three times more frequent than expected. Synonymous substitutions (10815 occurrences) were observed ten times more often than nonsynonymous substitutions (1060 occurrences).

Parsimony Analysis

The parsimony analysis shows polyphyly of the Old World vultures based on the cyt b dataset (Figure 1). *Sarcogyps, Trigonoceps* and *Aegypius* form a sister clade to *Necrosyrtes* and *Gyps.* There is strong support for the existence of two sister clades (95 boostrap value), but weak support for the placement of species within these clades. Circaetus remains part of an unresolved

polytomy not within this derived vulture clade. The remaining three vultures *Neophron,*

Gypaetus and *Gyophierax* form a much earlier diverging unresolved polytomy.

Bayesian Inference

The Bayesian analysis shows polyphyly of the Old World vultures based on the cyt b

dataset (Figure 2). *Neophron, Gypaetus* and *Gyophierax* form an early diverging monophyletic

clade sister to *Pernis*, while the other Old World vultures form a much more derived

monophyletic clade. Support for the early diverging clade of vultures is moderate (74 PP). The

more derived vulture clade consists of two sister groups, one formed by *Trigonoceps, Aegypius*

and *Sarcogyps*, and the other including *Gyps,* and *Necrosyrtes.* Support for the monophyly of this

clade is high (95 PP), as is support for the existence of two clades within the derived vulture clade

(100 PP) *Circaetus* is sister to the derived clade of vultures with moderate support (85 PP).

Discussion

Both the Bayesian and ML analyses support polyphyly of the Old World vultures based

on this cyt b dataset. The analyses agree that the more derived vultures form a monophyletic

clade consisting of two sister groups as found by Seibold and Helbig (1995). The first group

includes *Sarcogyps* as the earliest diverging species, then *Trigonoceps* and finally *Aegypius* as the

most derived species in this group. The second group has *Necrosyrtes* as sister to the *Gyps*

species. These results coincide for the most part with the anlyses done by Seibold and Helbig,

however, they support a sister relationship between Necrosyrtes and Gyps not found by Seibold

and Helbig.

The Bayesian analysis suggests that *Gypohierax, Neophron* and *Gypaetus* form a

monophyletic clade of early diverging vultures sister to a species long-supposed to be early

diverging: *Pernis.* The sister relationship between Gypaetus and Neophron was supported by

Seibold and Helbig's analysis and is reflected in shared morphological, embryonic development,

vocalizations and feeding behavior characteristics of the two species. Although Neophron and

Necrosyrtes have been considered closely related, they are best considered in terms of convergence according to this analysis (White, 1950).

The placement of *Circaetus* although unresolved in the ML analysis, is potentially sister to the derived vulture group as it is shown in the Bayesian analysis. This placement was proposed by Mundy et al. {, 1992}, although not supported by Seibold and Helbig's cyt b analysis. Mundy et al. also suggested a sister relationship for *Circaetus* and *Terathopius*. Both genera specialize on snakes as prey and have morphological adaptations for this lifestyle. Given the convergence seen between *Neophron* and *Necrosyrtes* in this analysis, it remains uncertain if *Circaetus* and *Terathopius* are sister or convergent species.

The placement of *Gypohierax* is supported strongly by the Bayesian analysis, as sister to the *Gypaetus-- Neophron* early diverging clade of vultures. A sister relationship between *Gypohierax* and *Neophron* was suggested by Brown and Amadon (1968), but has not been tested before with molecular evidence. Osteological evidence suggests a sister relationship between *Gypohierax,* and *Aegypius, Gyps*, and *Necrosyrtes* that was not supported with this cyt b dataset (Holdaway, 1994). If *Gyophierax* is truly sister to *Gypaetus* and *Neophron*, as suggested by this dataset, this relationship may help identify morphological characters that are convergent in many of the Old World vultures which have led to misplacement and confusion in previous phylogenetic analyses of vultures.

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