Molecular Phylogenetics of Diurnal Birds of Prey in the Avian Accipitridae Family

By

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Chapter 1

Introduction

Eagles, hawks, kites, accipiters and Old World vultures comprise the avian family Accipitridae. As ecologically sensitive predators, accipitrids are valuable indicators of habitat quality (Sergio *et al.*, 2005) and all accipitrid species are protected as CITES I or II species (IUCN, 2006). Traditionally recognized accipitrid species and subspecies vary morphologically from nearly indistinguishable to highly divergent, such that species and genera boundaries are not clear in many cases. Endemic populations within some species may warrant recognition as separate species or evolutionary units if diagnosable based on molecular data. A phylogeny, both within and among species and genera of Accipitridae, is therefore needed to delineate the genetic and overall biological diversity, which is of immediate concern to conservation efforts. A well-supported phylogeny of the Accipitridae can also provide insight into the evolution of the diverse accipitrid life-styles, and the biogeographic history of the family.

Previous phylogenetic studies using morphological (e.g. Holdaway, 1994; Kemp, Crowe, 1990; Kemp, Crowe, 1994) or molecular data (e.g. Bunce *et al.*, 2005; do Amaral *et al.*, 2006; Gamauf, Haring, 2004; Helbig *et al.*, 2005; Riesing *et al.*, 2003b; Seibold, Helbig, 1995) have produced incongruent results. Convergent morphology due to similar predatory lifestyles and morphological plasticity (Bunce *et al.*, 2005) has made morphological characteristics difficult to use in phylogenies, thus further investigations based on molecular datasets are needed.

In chapter two I evaluate relationships among all 14 previously described Accipitridae subfamilies with a molecular phylogeny based on 2087 bases of mitochondrial data and 1074 bases of nuclear data. Phylogenetic relationships within four subfamilies of eagles (booted eagles, sea eagles, harpy eagles and snake eagles) and two subfamilies of Old World vultures (Gypaetinae and Aegypiinae) are investigated with nearly complete taxonomic representation for these groups. In two species, *H.*

fasciatus and *H. morphnoides*, where subspecies are both morphologically distinct and separated by substantial geographical distance, I sampled multiple individuals of each subspecies to investigate their monophyly.

In chapter three I present a detailed analysis of the phylogenetic relationships within the accipitrid subfamily Buteoninae based on over 3000 bases of nuclear and mitochondrial DNA. Buteoninae is of particular interest as it comprises one of the largest accipitrid subgroups and includes multiple species of conservation concern. This study includes representatives of all genera previously included within or proposed as close relatives of the Buteoninae subgroup: *Buteo*, *Leucopternis*, *Buteogallus, Harpyhaliaetus*, *Busarellus*, *Parabuteo*, *Geranoaetus, Geranospiza, Ictinia, Rostrhamus, Kaupifalco* and *Butastur*. Multiple representatives of each nominal subspecies and species were included for three different "superspecies" complexes within the genus *Leucopternis* (*L. albicollis*, *L. polionotus* and *L. occidentalis; L. plumbeus* and *L. schistaceus*; and, *L. kuhli* and *L. melanops*).

In addition to establishing evolutionary relationships among and within subfamilies of Accipitridae, molecular sequence data is useful for identifying monophyletic groups for species delineation as shown in chapters two and three. In chapter four, fast-evolving sequences of DNA are used to evaluate levels of genetic diversity and population structure within a species where reciprocal monophyly is not present. With sequence data from 417 bases of the highly variable domain I of the mitochondrial control region for 66 harpy eagles (*Harpia harpyja*) sampled from across their broad geographic distribution this study uses a combination of test statistics and phylogenetic and coalescent-based analyses to assess levels of genetic diversity, population structure and demographic history for the harpy eagle. The harpy eagle (*Harpia harpyja*), the largest Neotropical bird of prey, is currently threatened by habitat loss, fragmentation and human persecution. Conservation and management programs, including captive-breeding, have been undertaken in multiple locations throughout the Neotropics (e.g. The Peregrine Fund in Panama, Parque Nacional Guayaquil in Ecuador and The Harpy Eagle Conservation Program in Brazil). The results of this study will be valuable for conservation efforts that aim to preserve genetic variability and retain

maintain historical levels of gene flow among geographic areas in the wild by quantifying existing levels of genetic diversity and identifying historic levls of gene flow.

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Chapter 2

Phylogeny of eagles, Old World vultures and other Accipitridae based on nuclear and mitochondrial DNA

Accipitridae is a diverse avian family, comprising up to 14 subfamilies, 65 genera and 231 species (see Table 1, Dickinson, 2003; Stresemann, Amadon, 1979). Of the Accipitridae species, some of the largest and most threatened by anthropogenic factors belong to four eagle subfamilies (Circaetinae, Haliaeetinae, Aquilinae and Harpiinae) and two Old World vulture subfamilies (Gypaetinae and Aegypiinae). All Accipitridae species are protected under the Convention on International Trade in Endangered Species (CITES) and four eagles are listed as top priority species (CITES I, CITES-Secretariat, 2003). As ecologically sensitive predators, birds of prey are valuable indicators of habitat quality. The Accipitridae are found in a variety of habitats from primary rainforest to arctic tundra throughout the world. Some taxa are restricted in distribution such as the snake eagles (Circaetinae) which are found only in the Old World, while others, such as the sea eagles (Haliaeetinae), are global in distribution. Thorough phylogenetic analyses are needed to delineate the genetic and overall biological diversity of this family, and to inform conservation programs which aim to preserve genetic diversity of distinguishable taxonomic units.

Table 1. Accipitridae subfamilies

Subfamily	Common Name	Genera	Brief Description	Genera placed in the subfamily by this analysis
Elaninae[1]	Kites	*Elanus, Gampsonyx, Chelictinia*	Kites noted for having a bony shelf above the eye, *Elanus* is cosmopolitan, *Gampsonyx* is restricted to the New World and *Chelictinia* is found in Africa	Elanus (*Gampsonyx* and *Chelictinia* not sampled)
Perninae[1]	Kites	*Pernis, Aviceda, Leptodon, Chondrohierax, Henicopernis, Elanoides, Machaerhamphus?*	Kites mainly found in the tropics and specializing on insects and bee or wasp larvae, all lack the bony eye shield found in the Elaninae	*Pernis, Leptodon, Chondrohierax, Elanoides, Hamirostra* and *Lophoictinia (Aviceda, Henicopernis and Machaerhamphus* not sampled*)*
Milvinae[1]	Milvine or Brahminy kites	*Milvus, Rostrhamus, Harpagus, Ictinia, Lophoictinia, Hamirostra, Haliastur*	Diverse kites found in the New and Old World, several species have fusion of joints of the second and third toes (Brown and Amadon, 1968)	*Milvus* and *Haliastur*
Aegypiinae[1].	Old World vultures	*Gyps, Pseudogyps, Necrosyrtes, Aegypius, Torgos, Trigonoceps, Sarcogyps*	Largest Old World vultures, scavengers, most with long necks and lightly feathered to bare heads	*Gyps/Pseudogyps, Necrosyrtes, Aegypius, Torgos, Trigonoceps* and *Sarcogyps*
Gypaetinae[2]	Old World vultures	*Neophron, Gypaetus, Gypohierax angolensis*	Generally smaller vultures found in the Old World with more restricted ranges, various specialized feeding behaviors, vocalizations, breeding	*Neophron, Gypaetus* and *Gyophierax*

			displays, *Gyophierax* and *Neophron* similar to each other in plumage coloration and molt stages	
Circaetinae[1]	Snake eagles	*Circaetus, Terathopius, Dryotriorchis, Eutriorchis, Spilornis*	Old World species feeding mainly on snakes, other reptiles and small mammals, have a reticulate pattern of heavy scales on the tarsi and relatively short toes	*Circaetus, Terathopius, Dryotriorchis, Spilornis* and *Pithecophaga*; sister relationship and subfamily for *Eutriorchis* undetermined here
Polyboroidinae[3]	Harrier Hawks	*Polyboroides, Geranospiza*	One New World and one Old World species, both exploit species found in tree cavities for prey, have short outer toe, increased mobility and length of the tarsus, relatively weak bill	*Polyboroides*
Aquilinae[2]	Booted eagles, hawk-eagles	*Aquila, Spizaetus, Hieraaetus, Stephanoaetus, Polemaetus, Ictinaetus* (considered a kite by Sushkin)*, Spizastur, Oroaetus*	Large eagles with feathered tarsi, globally distributed in diverse habitats taking a wide variety of prey, the hawk-eagles have crests	*Aquila, Spizaetus, Hieraaetus, Stephanoaetus, Polemaetus, Ictinaetus, Spizastur, Oroaetus,* and *Lophaetus*
Accipitrinae[2]	Sparrowhawks and (?) Chanting goshawks	*Accipiter, Urotriorchis, Megatriorchis, Erythrotriorchis, Melierax, Heterospizias* (?)	Small, fast fliers specializing on small birds as prey, long and slim tarsometatarsus and toes	*Accipiter* (*Urotriorchis, Megatriorchis, Erythrotriorchis* and *Heterospizias* not sampled)
Circinae [1, 6]	Harriers	*Circus*	broad and long-winged birds with facial feather disks, found mainly in open habitat such as fields or	*Circus*

			marshes, have specialized outer ears and related bones	
Haliaeetinae[4]	Sea and Fish eagles	*Haliaeetus, Ichthyophaga*	Large eagles found in riverine and coastal habitat throughout the world, all have fused basal joint of middle toe	*Haliaeetus, Ichthyophaga*
Buteoninae[2]	Hawks, buzzards, (usually includes booted eagles, sea eagles and harpy eagles which we have separated out here)	*Buteo*, *Geranoaetus, Parabuteo, Kaupifalco, Buteogallus, Harpyhaliaetus, Busarellus, Heterospizias* (?)*, Leucopternis, Butastur*	Predominately New World species of soaring hawks with long broad wings and relatively short tails and legs	*Buteo, Geranoaetus, Parabuteo, Buteogallus, Harpyhaliaetus, Leucopternis ,Ictinia, Geranospiza* and *Rostrhamus,* (*Busarellus, Heterospizias, Kaupifalco and Butastur* not sampled)
Harpiinae[5]	Harpy eagles	*Harpia, Morphnus, Harpyopsis, Pithecophaga, Harpyhaliaetus* (?)	Extremely large and powerful eagles with unfeathered tarsi, tropical forest predators of medium-sized mammals	*Harpia, Morphnus* and *Harpyopsis*
Melieraxinae[5, 6]	Chanting goshawks	*Melierax (Micronisus)*	Forest accipiters, larger than *Accipiter* species otherwise similar to that genus	*Melierax* (*Micronisus*)

[1]Peters 1931
[2]Gadow 1893
[3]Brown and Amadon 1968
[4]Sushkin 1905 in Jollie 1976
[5]this study
[6]Alternatively Circinae and Melieraxinae may be united under Accipitrinae with birds of the genus *Accipiter*

Phylogeny for Accipitridae based on morphological traits has been difficult to resolve (e.g. Brown, Amadon, 1968; Jollie, 1976; 1977a; 1977b). The few published molecular studies have been limited in sampling and have proposed some previously unrecognized relationships (see below). The goal of the present study is to identify phylogenetic relationships within and among the six subfamilies of eagles and Old World vultures in the context of the other primary accipitrid groups using molecular data.

The booted eagles (Aquilinae) are one of the largest accipitrid groups containing 35-36 species in 8-9 genera and are distributed worldwide. The majority of the species fall into three genera, *Aquila*, *Hieraaetus* and *Spizaetus*, while the remaining five genera are all monotypic. All species have "boots," or feathered tarsi, a trait that separates this group from most other accipitrid taxa. The booted eagles have been considered to be monophyletic (Jollie, 1977b) or polyphyletic (Holdaway, 1994) with morphological data, and only a few species in one genus have been studied phylogenetically with molecular data (Cyt-b, Seibold *et al*., 1996; control region, Vali, 2002). Monophyly of the three Aquilinae genera is not well-supported with morphological characters, such that the *Hieraaetus* species and some *Spizaetus* species have been placed in the genus *Aquila* by various authors (described by Brown, Amadon, 1968; and Thiollay, 1994). The two molecular studies included about half of the species in the genus *Aquila*, and both found that *A. chrysaetos* was genetically distant from four other *Aquila* species. Sister relationships for *A. clanga* and *A. pomarina, A. nipalensis* and *A. heliaca* or *A. heliaca* and *A. adalberti* were also proposed.

The sea eagles (Haliaeetinae) are a much smaller and more easily defined group of large eagles found in coastal and riverine areas worldwide except South America and

Antarctica. The two sea eagle genera, *Haliaeetus* and *Ichthyophaga*, share some morphological traits with two genera of kites (*Milvus* and *Haliastur*), suggesting a close relationship (Holdaway, 1994; Jollie, 1977b; Thiollay, 1994). The sea eagles also share some traits with the palmnut vulture (*Gypohierax angolensis*), suggesting a relationship between them and Old World vultures (Brown, Amadon, 1968). Using cyt-b sequence data, Seibold and Helbig (1996) studied eight of the nine species of sea eagles in the genus *Haliaeetus*. They supported a clear split between species with temperate versus tropical distributions, and a close relationship between the sea eagles and two *Milvus* kites. The relationship between the two genera of sea eagles has not been investigated with molecular sequence data and the possibility of paraphyly of the genera remains unresolved.

The four species and genera of harpy eagles (Harpiinae) are some of the largest raptors and are found in tropical rain forests in the Americas, the Philippines and New Guinea. This group is generally considered monophyletic due to their large size, lack of feathers on the tarsi and similarities in behavior (Brown, Amadon, 1968; Thiollay, 1994); however, some have suggested that the Old World species are not sister to the New World species (e.g. Jollie, 1977b). Holdaway (1994) removed one Old World (*Pithecophaga*) and one New World (*Morphnus*) species from the Harpiinae. A close relationship between the booted eagles and the harpy eagles has been proposed but not tested with molecular data.

The 14 species of snake eagles (Circaetinae) in five genera are found only in the Old World. Although usually considered monophyletic (Brown, Amadon, 1968; Friedmann, 1950a), the possibility of polyphyletic origins for snake eagles has been

raised (Jollie, 1977b could not identify sister relationships for Eutriorchis and Dryotriorchis; Thiollay, 1994).

The final group we focused on is the Old World vultures, a diverse mix of scavengers including at least one species that uses tools (Egyptian vulture, *Neophron percnopterus*), and potentially including a frugivorous raptor (palmnut vulture, *Gypohierax angolensis*). One or two subfamilies have been proposed for the Old World vultures. Three species are highly divergent from the remaining 11 and have been placed by some in a separate family called Gypaetinae (Mundy *et al.*, 1992). The core 11 species are called the Aegypiinae. Seibold and Helbig (1995) used cyt-b sequence from eleven Old World vulture species and found evidence of polyphyly for the Old World vultures.

There are no previously published molecular studies that include representatives of all of the Accipitridae subfamilies; however, several molecular studies have used mitochondrial DNA to examine particular Accipitridae subgroups and have found evidence for polyphyly of some traditionally recognized taxa (and the genus Buteo, Gamauf, Haring, 2004; e.g., polyphyly of the Perninae kites, Riesing *et al.*, 2003a). Relationships among a small set of accipitrids based on mtDNA indicated a closer relationship between a representative sea eagle and kite in the genus *Milvus*, than between the sea eagle and a snake eagle in the genus *Circaetus*. A representative Old World vulture was more closely related to the snake eagle than other accipitrid taxa in the study, including species of *Buteo, Haliaeetus, Milvus, Circus, Accipiter,* and *Pernis* (Mindell et al. 1997). Increased sampling of species and molecular characters are needed to improve our understanding of phylogenetic relationships among the Accipitridae.

In this study we focus on full or nearly complete taxonomic representation of five accipitrid subgroups (sea and fish eagles, harpy eagles, booted eagles, snake eagles and Old World vultures), corresponding to six potential subfamilies. We use both mitochondrial and nuclear sequences for representatives of 51 out of 65 genera (78%) and just under half of the known Accipitridae species (n=111). At least one representative of each previously proposed subgroup/subfamily within the Accipitridae have been included to help in phylogenetic placement of the focal taxa.

Methods

Taxon sampling.—We include at least one representative from all genera and the majority of species of sea and fish eagles (2 genera, 10 species), snake eagles (4 genera, 12 species), harpy eagles (4 genera, 4 species), booted eagles (8 genera, 29 species) and Old World vultures (9 genera, 13 species), based on the taxonomy in Dickinson (2003). In two cases where significant morphological differences among geographical populations have been documented, multiple samples representing different subspecies were included in the analysis. To infer relationships among these subfamilies within the Accipitridae we also include at least one representative from each primary group or clade within the Accipitridae family as proposed by Gadow (1893), Peters (1931), Brown and Amadon (1968), Jollie (1977b), Stresemann and Amadon (1979) and Holdaway (1994). *Falco longipennis, Falco peregrinus,* and *Phalcoboenus megalopterus* (Falconidae) were used as outgroup taxa. Samples, their sources and locality information are listed in Table 2.

Table 2. List of Taxa and Samples Used for DNA Sequencing

Order Family Subfamily	Species	Locality	Source[a] and Voucher # [b]	Tissue ID
Falconiformes				
Falconidae	*Falco peregrinus*	N. America		
	Falco longipennis	Australia	AM-EBU	10665
	Phalcoboenus megalopterus	South Africa	Captive, WOB, P	WOB-3
Sagiitaridae	*Sagittarius serpentarius*	South Africa	Captive, JBZ, P	JBZ-12
Pandionidae	*Pandion haliaetus*	Michigan	UMMZ 225997	T-264
Accipitridae	*Elanus leucurus*	South Africa	Captive, CRH, P	CRH-4
Elaninae				
	Polyboroides typus	Gambia, Africa	UMMZ 235187	T-1423
Polyboroidinae				
Gypaetinae	*Neophron percnopterus*		DWC, Captive, P	DWC-1
	Gypohierax angolensis	Gambia, Africa	UMMZ 235794	A-1232
	Gypaetus barbatus		Captive, SDZ	
	Eutriorchis astur	Madagascar	TPF, Wild, P	
Perninae	*Chondrohierax uncinatus*	Grenada	TPF, Wild, P	
	Leptodon cayanensis	Paraguay	KUNHM	139
	Elanoides forficatus	Ecuador	LSUMNS	B-12133
	Pernis apivorus		TAU	
	Hamirostra melanosternon	Australia	AM-EBU	1
	Lophoictinia isura	Australia	AM-EBU	0.50591
	Pithecophaga jefferyi	The Philippines	TPEF, captive	

Circaetinae				
	Pithecophaga jefferyi	The Philippines	TPEF, captive	
	Pithecophaga jefferyi	Mindanao, Philippines	AMNH 534856	
	Terathopius ecaudatus	South Africa	UBP, Captive	UMG-3
	Spilornis elgini	S. Andamens	NHM-UK 1885.8.19.1626	
	Spilornis holospilus	Mount Calavite, Occ. Mindoro	AMNH 784054	
	Spilornis cheela burmanicus	Cherrapunji, India	UMMZ 140566	
	Spilornis rufipectus	S. Celebes	AMNH 536566	
	Circaetus pectoralis	South Africa	Captive, PBC, P	PBC-3
	Circaetus gallicus		TAU	363
	Circaetus cinereus	Zambia	UMMZ	A752
	Dryotriorchis spectabilis	Eastern Congo Forest, Africa	AMNH 448333	
	Circaetus fasciolatus	South Africa	WOB, Captive, P	WOB-3
	Circaetus cinerascens	Karonga, Nyasaland	NHM-UK 1948.26.1	
	Necrosyrtes monachus	Gambia	UMMZ	A1234
Aegypiinae				
	Gyps bengalensis		TPF	
	Gyps rueppellii	Gambia	UMMZ	A1119
	Gyps fulvus	Gambia	UMMZ 235890	B19181
	Gyps coprotheres	South Africa	DWC, Captive, P	DWC-10
	Gyps africanus		TAU	
	Sarcogyps calvus	South Africa	DWC, Captive, P	DWC-20
	Trigonoceps occipitalis	Senegal	UMMZ 130316	
	Aegypius monachus		DZ, Captive, P	1903

	Torgos tracheliotus		UMMZ 234705	T-2046
Harpiinae	*Harpyopsis novaeguineae*	New Guinea, Southern Highlands Province, Piambil Village, Mt.Giluue	UMMZ	238858
	Morphnus guianensis	Peru	HUA, Captive, P	HUA-19
	Harpia harpyja	Colombia	Captive, SDZ	402158
Aquilinae	*Spizaetus lanceolatus*	Celebes	NHM-UK 1887.11.1.337	
	Spizaetus cirrhatus lineatus	Bamanigaon, Assam, India	UMMZ 140516	
	Spizaetus nanus	Lambuk River, Central North Borneo	NHM-UK 1956.60.11	
	Spizaetus nipalensis		NBPC, Captive, P	
	Spizaetus alboniger	Gomantong, North Borneo	NHM-UK 1956.60.9	
	Spizaetus tyrannus	Peru	HUA, Captive, P	HUA-25
	Spizastur melanoleucus	Peru	HUA, Captive, P	HUA-28
	Spizaetus ornatus	Darien Province, Panama	LSU	B2267
	Oroaetus isidori	Peru	HUA, Captive, P	HUA-23
	Stephanoaetus coronatus	South Africa	PBC, Captive, P	PBC-9
	Hieraaetus kienerii		NHM-UK 1877.85.8.19.1331	
	Polemaetus bellicosus	South Africa	EES, Captive, P	EES-1
	Lophaetus occipitalis	South Africa	PBC, Captive, P	PBC-15
	Ictinaetus malayensis malayensis		NHM-UK 1932.12.21.-35	
	Hieraaetus pennatus	South Africa	WOB, Captive, P	WOB-5

	Hieraaetus pennatus	Punjab, India	UMMZ 75313	
	Hieraaetus morphnoides morphnoides		UMMZ	T-2796
	Hieraaetus morphnoides morphnoides	Australia	NHM-UK 1969.4.22	
	Hieraaetus morphnoides weiskei	New Guinea	AMNH 535061	
	Hieraaetus morphnoides weiskei	New Guinea	NHM-UK 1913.3.6.35	
	Hieraaetus ayresii	Uganda, Africa	UMMZ 535074	
	Aquila wahlbergi		DWC, Captive, P	DWC-21
	Aquila chrysaetos	N. America	UMMZ	238855
	Spizaetus africanus		NHM-UK 1977.20.43	
	Hieraaetus fasciatus fasciatus	Red Sea, Egypt	UMMZ 224053	
	Hieraaetus fasciatus fasciatus	Bhadwar, India	UMMZ 78295	
	Hieraaetus fasciatus fasciatus	Parwali, India	UMMZ 78294	
	Hieraaetus fasciatus spilogaster	South Africa	WOB, Captive, P	WOB-13
	Hieraaetus fasciatus spilogaster	South Africa	EES, Captive, P	EES-3
	Aquila verreauxii	South Africa	PBC, Captive, P	PBC-8
	Aquila audax	Moomba, South Australia	SAM, SAMAB48364	ABTC-02866
	Aquila gurneyi	Halmahera, Indonesia	NHM-UK 1873.5.9.8	
Melieraxinae	*Melierax gabar*	Zimbabwe	UMMZ	A765
Circinae	*Circus aeruginosus*		TAU	353
	Circus ranivorus	South Africa	Captive, PBC-6, P	PBC-6
Accipitrinae	*Accipiter bicolor*	Santa Cruz Dept., Bolivia	LSU	B-18875
	Accipiter cooperii	Michigan, U.S.A.	UMMZ 227082	T-293
Milvinae	*Haliastur indus girenera*	Brunswick Heads, Australia	AM-EBU, 064910	EBU 11377

	Haliastur sphenurus	Gregory, Northern Territory, Australia	SAM, NTMT651	ABTC-27746
	Milvus migrans parasitus	Cameroon, Africa	AMNH 388140	
	Milvus milvus	Rome, Italy	AMNH 531856	
	Haliaeetus leucoryphus	Palasbari, India	UMMZ 142065	
Haliaeetinae				
	Haliaeetus pelagicus		NBPC, Captive, P	JPJ MB 26
	Haliaeetus albicilla		TAU	
	Haliaeetus leucocephalus	N. America	UMRC	N42
	Ichthyophaga humilis	Bhadwar, India	UMMZ 78356	
	Ichthyophaga ichthyaetus	Palasbari, India	UMMZ 140540	
	Haliaeetus vocifer	Durban, South Africa	UMMZ	A1075
	Haliaeetus vociferoides	Madagascar	R. Tingay	MFE 60 0051
	Haliaeetus leucogaster	Lincoln, South Australia	SAM, SAMAB48773	ABTC 03064
	Haliaeetus sanfordi	Solomon Islands	UMMZ 112326	
	Ictinia plumbea	Paraguay	KUNHM	2900
Buteoninae				
	Geranospiza caerulescens	Paraguay	KUNHM	3110
	Rostrhamus sociabilis	Guyana	KUNHM	5852
	Buteogallus urubitinga	Paraguay	UMMZ 227470	SMG 2546
	Harpyhaliaetus coronatus	Capitan Bado, Paraguay	UMMZ 101669	
	Harpyhaliaetus solitarius	Peru	HUA, Captive, P	HUA-18
	Buteo magnirostris	Loreto Dept., Peru	LSU	B-2862
	Parabuteo unicinctus	Arizona, U.S.A.	UMMZ	T-1039
	Geranoaetus melanoleucus	Peru	HUA, Captive, P	HUA-03
	Leucopternis albicollis	Tigre Playa, Sucumbios,	ZMUC 114919	

	Ecuador		
Buteo buteo		Genbank	
Buteo jamaicensis	N. America	UMMZ	T-2797
Leucopternis kuhli	Loreto Dept., Peru	LSU	B-4598
Leucopternis melanops	Loreto Dept., Peru	LSU	B-7167

[a] AM-EBU, Australian Museum Evolutionary Biology Unit, Sydney, Australia; CRH, Center for Rehabilitation of Wildlife, South Africa; DWC, De Wildt Cheetah and Wildlife Reserve, Pretoria, South Africa; DZ, Detroit Zoo, Detroit, MI; EES, Eagle Encounters at Spier, Stellenbasch, South Africa; HUA, El Huayco, Peru; JBZ, Johannesburg Zoo, South Africa; KUNHM, Kansas University Natural History Museum; LSUMNS, Louisiana State University Museum of Natural Science; NBPC, National Birds of Prey Centre, Newent, England; NHM-UK, The Natural History Museum, Tring, United Kingdom; PBC, Predatory Bird Centre, South Africa; SAM, South Australia Museum, Adelaide, Australia; SDZ, San Diego Zoo, CA; TAU, Tel Aviv University Research Zoo; TPEF, The Philippine Eagle Foundation, The Philippines; TPF, The Peregrine Fund; UBP, Umgeni Bird Park, South Africa; UMMZ, University of Michigan Museum of Zoology, Ann Arbor, MI; UMRC, University of Minnesota Raptor Center, MN; WOB, World of Birds, Cape Town, South Africa;

[b] When a live bird was sampled, a photo was taken at the time of sample collection. Availability of a photo is signified by the letter "P." When a museum skin was sampled only the voucher number is given, no tissue number is given.

Sequencing.—Genomic DNA was extracted from muscle tissue or blood using proteinase K digestion following the manufacturer's protocols (DNeasy tissue kit, Qiagen), or from the calamus of primary feathers by adding dithiothreitol (30 ml of 100 mg/ml, Cooper, 1994) to the overnight tissue digestion buffer, and then proceeding according to the manufacturer's protocols. For museum skin (toe pad) samples, genomic DNA was extracted from toe pad tissue digested overnight as described for feathers above, with additional washes of 500ul Salton Wash 1 and Salton Wash 2 (Qbiogene, Inc.).

All museum toe pad extractions and PCR preparations were conducted in a facility exclusively designated for old/degraded DNA samples at the University of Michigan Museum of Zoology and the Ancient Biomolecules Centre (ABC) at Oxford University. To prevent contamination, no contemporary samples or PCR products are permitted in either facility (Cooper, Poinar, 2000).

We sequenced 1047 bases of mitochondrial NADH dehydrogenase subunit 2 (ND2), 1041 bases of mitochondrial Cytochrome-b (cyt-b) and 1074 bases of nuclear Beta-fibrinogen intron 7 (BF-I7) in segments of ~250 to 1080 bases in length. Primers used are described in Table 3. PCR products were visualized on a 1% low melting point agarose gel stained with ethidium bromide and gel extracted with a Gel Purification kit (Qiagen). Sequencing was performed on an ABI Model 3730 sequencer. Resulting chromatographs for both strands of DNA were resolved in Sequencher version 4.1.

We took standard precautions against inadvertent amplification of nuclear copies of mitochondrial genes (see Arctander, 1995; Mindell *et al.*, 1997; Sorenson, Fleischer, 1996). In cases where double peaks on chromatographs identified potential multiple copies for ND2, we cloned the PCR products using a TOPO^{TM} TA Cloning kit

(Invitrogen), and sequenced 5 clones to identify separate DNA sequences. Two sequences of identical length lacking internal stop codons were found in multiple clones of the *Morphnus guianensis* PCR product. Both clones were included in the analyses.

Dataset construction and analyses.—Sequences were aligned in BioEdit v. 7.0.0 (Hall, 1999a) by eye. Cyt-b did not contain indels and the indels found in ND2 and BF-I7 were easily resolved. Nine vulture species had an insertion of two amino acids immediately preceding the stop codon of ND2 (*Aegypius monachus, Torgos tracheliotus, Gyps africanus, G. bengalensis, G. coprotheres, G. fulvus, G. rueppellii, Sarcogyps calvus,* and *Trigonoceps occipitalis*). In BF-I7 the two *Haliastur* species shared an insertion of one base and the two *Accipiter* species shared an insertion of one base and a separate insertion two bases in length. An insertion of twelve bases was found in three *Falco* species (*Falco longipennis*, *F. subniger and F. biarmicus*, though the last species was not included in further analyses) and eleven separate insertions were autapomorphic. The two *Buteogallus* species and *Geranospiza caerulescens* share a deletion of two bases; the two *Buteogallus* species and *Harpyhaliaetus solitarius* share another separate deletion of two bases; the three *Falco* species share a deletion of one base; the two *Harpyhaliaetus* species share a deletion of two bases; *Harpia harpyja, Harpyopsis novaeguineae* and *Morphnus guianensis* share a deletion of eight bases; and, *Harpyhaliaetus solitarius, Leptodon cayanensis* and *Rostrhamus sociabilis* share a deletion of two bases.

Table 3. Primers used in study to amplify mitochondrial and nuclear gene regions

Gene and Target group	Primer Name	Sequence (5-3') or Reference
ND2 all species	L5219, H5766, L5758, H6313	Sorenson et al. (1999)
ND2 Booted eagle museum skins	H5501.eagle	TGA TAT YTC ATT GGC CDG TRG
	L5367.eagle	CAA CAC BCT YGC YAT CAT CC
	L5418.eagle	CAT YGA RGC YAC WAT CAA RT
	H5755.eagle	ABT TTT CGR AGT TGB GTT TG
	H6044.eagle	TGG ATR AYR AGY CAT TTR GGT A
	L5700.eagle	YCA CTC VCT YAA YCC DAC AYT
	L5971.eagle	TCH CCH HCA CTA AAY GCA AC
ND2 Snake eagle museum skins	H5592.snake	TCT GGG AAT CAG AAG TGR AAG
	L5513.snake	GRG AYA TYA CCC AAC TAAC C
	H5906.snake	GGT GAG TTT RGG RYT GTA GA
	L5768.snake	GRT GAA TRG GCC TAA ACC AAA
	H6133.snake	GCG AGR CGG AGG TAG AAG AA
	L6001.snake	GTC CTA CTY TCY CTA GCA GGR CTC
ND2 Sea eagle Museum skins	H299.cvk	Johnson et al. (in review)
	L247.cvk	Johnson et al. (in review)
	H852	Johnson et al. (in review)
	L768.cvk	Johnson et al. (in review)
Cyt-b all species	L14996, H15646, L15560, H16064	Sorenson et al. (1999)
Cyt-b Booted eagle museum skins	H15334.eagle	GAC TGT DGY CCT CAR AAR G
	L15244.eagle	YAA RGA RAC CTG AAA YACA GGA
	H15588.eagle	TCC YAR RRT RTC TTT TAR GGA GAA
	L15515.eagle	CYT DCA CGA RTC HGG VTC HA
	H15851.eagle	CGR AAD GTT ATT GTD CGY TG
	L15739.eagle	CCT ATT YGC ATA YGC BAT YC
	eagle-cytb-1f	Bunce et al. ((in press))
	eagle-cytb-3r	Bunce et al. (in press)
Cyt-b Snake eagle museum skins	H15310.snake	TTG GCC TCA TGG YAG GAC RT
	L15519.snake	CAC GAA WCH RGC TCA AAC AA
	H15587.snake	CCT AGR ATR TCT TTT ARR GAG AA
	H16020.snake	TTC TAG YGC YCC RGY TAG

Cyt-b Sea eagle museum skins	H15332.cvk	(Johnson *et al.*, in press)
	L15279.cvk	(Johnson et al, in review)
	L15560.cvk	(Johnson et al, in review)
	H15828.cvk	(Johnson et al, in review)
	L15748.cvk	(Johnson et al, in review)

Phylogenetic analyses were first performed on the individual genes to assess congruence of the phylogenetic signal among genes. Then the data were combined into two data sets for final analyses: one dataset includes 2088 bases of ND2 and cyt-b for 113 taxa and is referred to hereafter as the "mt dataset;" the other dataset includes the 2088 bases of mitochondrial data from the first dataset, plus an additional 1074 bases of nuclear data (BF-I7) for 71 taxa and is hereafter referred to as the "nuc + mt dataset."

Homoplasy and heterogeneity of base composition are two factors that, if not addressed in the phylogenetic model, may confound analyses. We tested our data for saturation at each codon position as a measure of homoplasy. Saturation plots (not shown) were constructed for each gene from the data matrix produced in DAMBE version 4.213 (Xia, 2000) using Tamura-Nei genetic distance and pairwise numbers of transitions and transversions. Saturation of codon position three in both ND2 and cyt-b was observed. Codon positions one and two did not show significant saturation in either mitochondrial gene. All base positions were analyzed together for BF-I7 as it does not encode protein, and no evidence of saturation was identified for BF-I7. We also tested for skewness in base composition as implemented in PAUP* 4.0b10 (Swofford, 2004) and found no significant departure from homogeneous base composition in both the mt and nuc + mt datasets.

To reconstruct phylogenies we used both maximum parsimony (MP) as implemented in PAUP* 4.0b10 (Swofford, 2004) and Bayesian inference using Markov chain Monte Carlo in the program MrBayes 3.01 (Huelsenbeck, Ronquist, 2001). MP analyses were heuristic with starting trees obtained by random addition of taxa with 100 replicate searches and TBR branch swapping. Successive analyses were done with all characters equally weighted, with a transition:transversion ratio of 5:1 for mitochondrial data and 2:1 for the nuclear data. These values were obtained by estimating the transition:transversion ratios from the alignments and from preliminary trees. The data were resampled using 500 bootstrap replicates to determine support at each node.

Models of evolution for parameter estimation and likelihood analysis were determined using the hierarchical log-likelihood ratio tests in the programs MrModelTest (Nylander, 2002) and DT ModSel (Minin et al. 2003). The simplest best-fit model for the two mitochondrial genes (analyzed separately) was GTR + I + G. Therefore, the mt dataset was not partitioned by gene as the model selected independently for both genes was the same. Third codon positions were unlinked from first and second positions to minimize the effect of saturation. We ran four Markov Chains in the program MrBayes for six million generations (mt dataset only), sampling every 500 generations for each dataset.

For the nuclear sequences DTModSel and MrModelTest both identified the GTR + G as the simplest best-fit model. The combined nuc + mt dataset was partitioned for Bayesian analyses so that the best-fit models were applied separately to the mitochondrial and nuclear data, and mitochondrial codon positions were all unlinked from each other.

We ran four Markov Chains for four million generations, sampling every 500 generations.

Analyses of both datasets were performed independently three times from random starting points so that convergence of topology and log-likelihood scores could be evaluated. Parameter stationarity was visualized in the program Tracer (Rambaut, Drummond, 2003). All three Bayesian runs of the mt dataset reached stationarity in all substitution model parameters and likelihood scores prior to 400,000 generations and a slightly more conservative burn-in time of 600,000 generations was used. The three Bayesian runs of the nuc + mt dataset reached stationarity in all substitution model parameters and likelihood scores prior to 200,000 generations and a conservative burn-in time of 400,000 generations was used. The tree topologies produced from the three separate runs of each dataset were identical in topology, only varying slightly in support values for nodes (<0.02 difference among Bayesian posterior probabilities).

Results

Gene properties: sequence composition and divergence.—We sequenced 1047 bases of ND2 and 1041 bases of cyt-b for 110 individuals representing 106-108 species and 1074 bases of BF-I7 for 68 of the same 106-108 species. ND2 contained the most variable sites, the most parsimony informative sites, the highest transition-transversion ratio and had a higher maximum divergence among species as compared to BF-I7 and cyt-b (Table 4). BF-I7 had the lowest percent divergence among taxa and the lowest transition – transversion ratio of the three sequences. Cyt-b had the highest G-C content. Consistency and retention indices are reported for each gene although such measures are not predictive of the ability of the gene to infer the correct tree topology (Simmons *et al.*, 2004).

Table 4. Sequence composition and divergence

	Total bases	# Variable sites/%	# Parsimony informative sites/%	% G-C	Maximum %/ Minimum % divergence between taxa	Consistency Index (nuc + mt dataset)	Retention Index (nuc + mt dataset)	Ti:tv ratio
BF-I7	1074	434/40.4	208/19.4	36.77	0.00016/16.8	0.8045	0.8264	1.93
Cyt-b	1041	504/48.4	462/44.3	47.84	0.23/23.9	0.2900	0.5915	5.03
ND2	1047	658/62.8	589/56.3	46.15	0.31/38.6	0.3280	0.5538	4.61

Phylogenetic analyses.—Two to three species of Falconidae were used as outgroup taxa. Some initial analyses were performed using two Musophagiformes as outgroups: *Crinifer piscator* and *Musophaga violacea.* However, this did not alter the results, and trees rooted with Falconidae species are shown here given the existing evidence for a close relationship between the Accipitridae and Falconidae (Mindell *et al.*, 1997; Seibold, Helbig, 1995). We also included the secretarybird (*Sagittarius serpentarius*) and the osprey (*Pandion haliaetus*) to help reduce any long branches between accipitrids and the falconid outgroup.

Both datasets contained at least one representative of every major Accipitridae taxon or clade previously proposed. We used preserved museum skins where fresh tissue, blood or feathers was not available. DNA in museum skins is more degraded than in fresh tissue, requiring amplification of at least two to four times the number of overlapping regions per gene. Nuclear DNAs are already at a lower concentration than mitochondrial DNAs in bird tissues, increasing the difficulty of amplification of nuclear sequences from museum skins. We attempted to amplify four regions of nuclear BF-I7 for five museum skins of which four amplifications of two regions were successful despite several attempts. Given the lower variability of BF-I7 and the increased amount of work and cost, we did not pursue nuclear sequence for all museum skins but instead focused on representing each major subgroup/subfamily of Accipitridae in both datasets and all species of eagles and vultures in the mt dataset. We also added taxa to our initial analyses to break up long branches among Perninae and Gypaetinae species. While the increased taxon sampling did serve to break up some of those long branches, the longest

branches in both analyses, aside from the Elaninae and other families or outgroups, are still found in the early-diverging Perninae clade.

Analyses using Bayesian inference of the two different datasets recovered identical tree topologies (Figures 1 and 2). The tree topology in Figure 1 was recovered by three independent Bayesian analyses of the mitochondrial dataset and the topology presented in Figure 2 resulted from 3 independent Bayesian runs using the nuc + mt dataset. The average Bayesian posterior probability for each node, and bootstrap values for clades corresponding to those recovered in the parsimony analysis are shown on each tree.

The MP analysis of the mitochondrial dataset found three shortest trees, each 18847 steps in length. There was one polytomy present in the final MP bootstrap tree (not shown). Resolved branching patterns followed the topology recovered in the Bayesian analyses with the following minor discrepancies. First, MP analysis was not able to resolve the branching pattern within the earliest diverging clade of kites and vultures beyond the sister relationships between Chondrohierax uncinatus and Leptodon cayanensis, Gypaetus barbatus and Neophron percnopterus, and Hamirostra melanosternon and Lophoictinia isura. A sister relationship between *Pithecophaga jefferyi* and *Terathopius ecaudatus* was recovered with very low bootstrap support (bs = 52) by MP. In the MP topology *Necrosyrtes monachus* was not sister to the Gyps species, but formed the first branch splitting from the clade containing *Sarcogyps* (bs=54). Finally, branching patterns within the Buteoninae clade including hawks (*Leucopternis*, *Geranoaetus*, *Buteo*, *Geranospiza* and *Parabuteo*) and kites (*Ictinia* and *Rostrhamus*) differed slightly in the two analyses, however both recovered a topology

Figure 1. Phylogeny for Accipitridae taxa inferred from mitochondrial cyt-b and ND2 sequences. Topology shown is the Bayesian inference majority rule tree (see text for details). Bayesian posterior probability values are shown above branches and MP bootstrap values (>50%) are shown in italics below the branches.

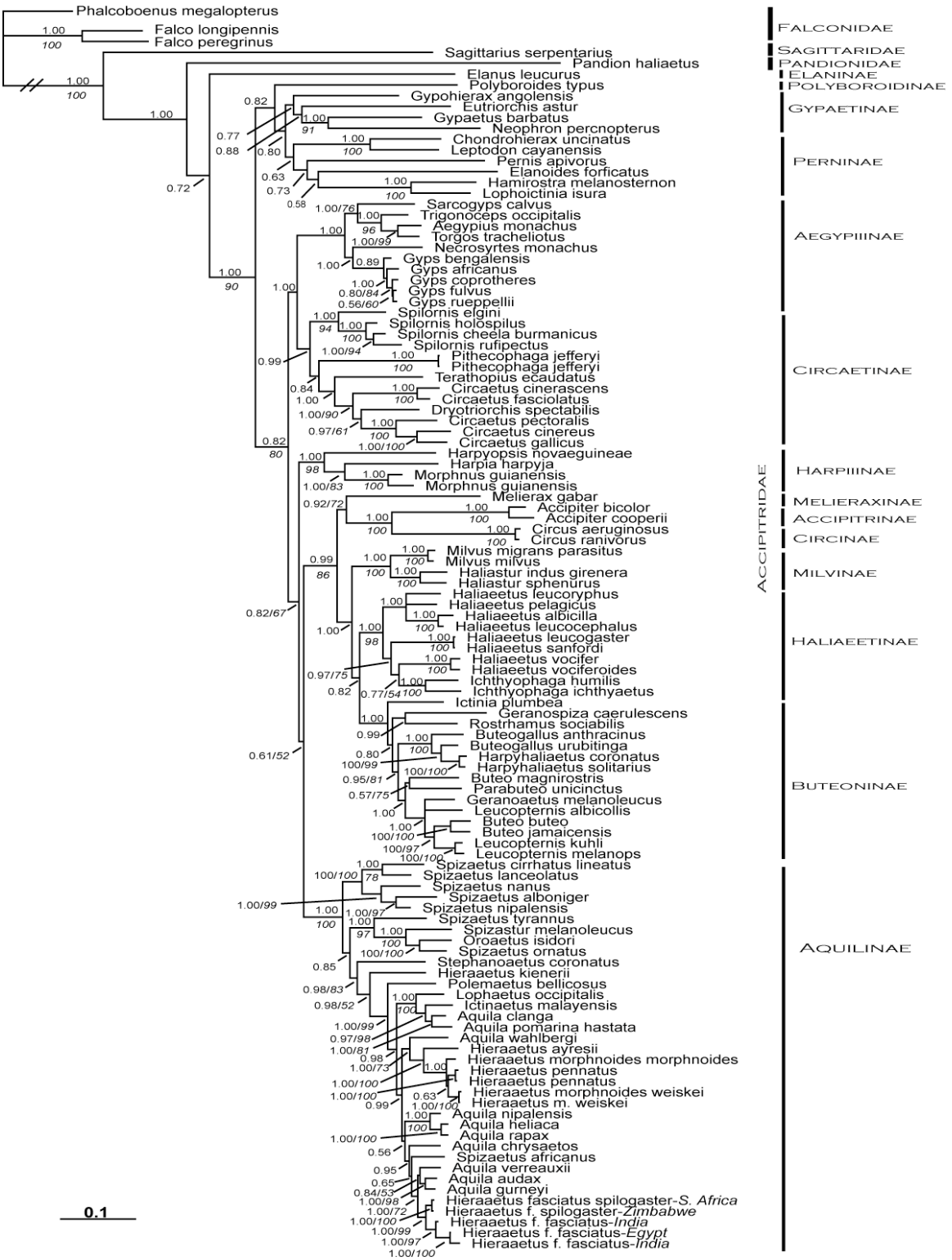
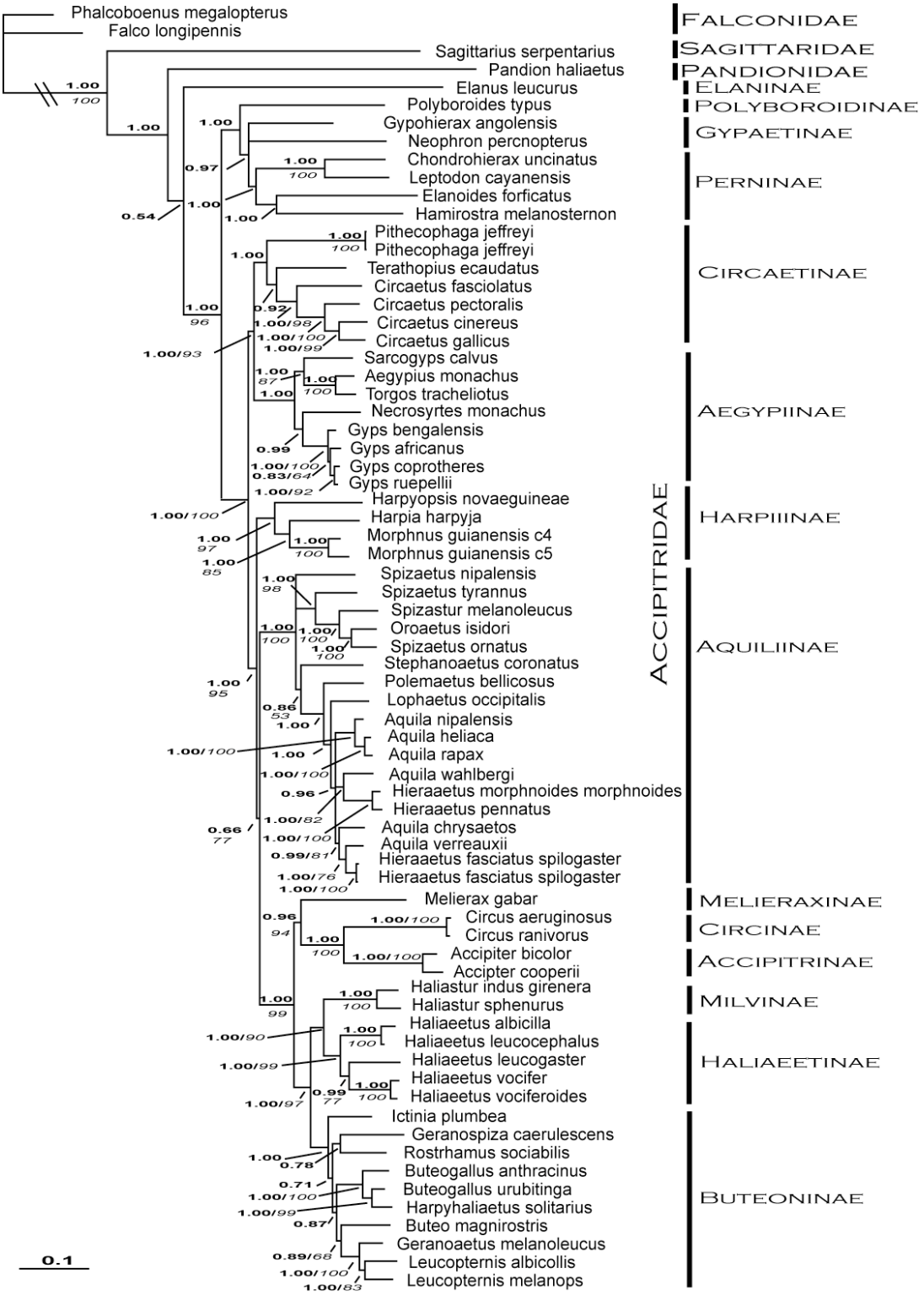


Figure 2. Phylogeny of the Accipitridae inferred from mitochondrial cyt-b and ND2 and nuclear Beta-fibrinogen intron 7 sequences. Topology shown is the Bayesian inference majority rule tree (see text for details). Bayesian posterior probability values are shown above branches and MP bootstrap values (>50%) are shown in italics below branches.



where the two *Harpyhaliaetus* species are nested within a clade of two *Buteogallus* species. The relationships within the Buteoninae aside from the *Harpyhaliaetus* species are not the focus of this paper and will not be addressed further here.

The MP analysis of the nuc + mt dataset with all characters equally weighted, gaps as a 5th state and a transition-transversion ratio (reflecting their relative frequencies) of 5:1 for mitochondrial genes and 2:1 for BF-I7 found three best trees of length 15306. Resolved branching patterns followed the topology recovered in the Bayesian analyses and bootstrap values are shown on the Bayesian consensus tree for resolved nodes (Figure 2). As found in the MP analysis of the mt dataset, in the nuc + mt MP analysis relationships among species in the earliest diverging kite/vulture clade were unresolved, *Pithecophaga jefferyi* and *Terathopius ecaudatus* were sister with low support (bs=55) and the position for *Necrosyrtes monachus* was unresolved.

Phylogeny of Accipitridae (Combined results from all datasets and analyses).—Both datasets and all analyses support monophyly for two of the four eagle groups: sea eagles (Haliaeetinae) and booted eagles (Aquilinae). Monophyly of the harpy eagle group (Harpiinae), the snake eagle group (Circaetinae) and the Old World vultures, however, is not supported in any of the analyses. Topologies within these groups are discussed in detail below. Where Bayesian posterior probabilities (PP) and parsimony bootstrap values (bs) are shown in the text, the value from the mitochondrial dataset is listed first followed by the value from the nuclear dataset when available.

Several other Accipitridae genera and subfamilies are also polyphyletic in our analyses. Three separate clades of kite species (Elaninae, Perninae and Milvinae)

proposed by morphological data were identified, however the Milvinae and Perninae subfamilies are polyphyletic. Two other kite species (*Ictinia plumbea* and *Rostrhamus sociabilis*) were more closely related to buteonine taxa than to other kites and did not fall into any of the traditional kite subfamilies. *Polyboroides typus* and *Geranospiza caerulescens* were not closely related to each other. The genus *Buteo* was polyphyletic with the roadside hawk (*Buteo magnirostris*) not sister to two other *Buteo* species. The genus *Buteogallus* was also polyphyletic, with two *Harpyhaliaetus* species nested within the genus.

Booted eagles (Aquilinae).—While the large booted eagle subfamily forms a well-supported monophyletic group with high Bayesian posterior probability (PP=1.00, 1.00) and high bootstrap values (bs=100, 100) with respect to other Accipitridae groups in all analyses, three genera within this group are not monophyletic: *Spizaetus*, *Aquila* and *Hieraaetus*. Forcing monophyly of the genus *Spizaetus* in the mt dataset adds 226 parsimony steps to the shortest tree of length 18847 (all such topological constraints in the following text refer to the MP analysis of the mt dataset). Members of the genus *Aquila* are found in three of six main clades in the booted eagle group. To force monophyly of the genus *Aquila* an additional 103 parsimony steps are needed. Species in the genus *Hieraaetus* are placed in the two latest diverging clades of booted eagles and one species forms a separate early-diverging clade by itself. Forcing monophyly of the genus *Hieraaetus* requires 84 additional parsimony steps

Hieraaetus f. fasciatus and *H. f. spilogaster* have been treated variously as separate species (Ferguson-Lees, Christie, 2001; Thiollay, 1994), a superspecies

(Stresemann, Amadon, 1979) or subspecies (Sinclair *et al.*, 2002). Here we sampled two *H. f. fasciatus* individuals from India, one *H. f. fasciatus* individual from Egypt, two *H. f. spilogaster* individuals from South Africa and one *H. f. spilogaster* from Zimbabwe. The three Indian and Egyptian samples shared identical cyt-b sequence except for one Indian sample at one base (sequence identity = 98.9%). The three *H. f. spilogaster* individuals from South Africa and Zimbabwe were different from the three other samples at 16 base positions in cyt-b (sequence identity = 90.2%) and another 18 bases in ND2 (sequence identity = 93.2%).

Two individuals of each *Hieraaetus morphnoides* subspecies were sampled: *H. m. morphnoides* and *H. m. weiskei*. Additionally two *H. pennatus* individuals from disparate locales were sequenced. All *H. pennatus* individuals shared identical ND2 and cyt-b sequences. *H. m. weiskei* samples also had identical mitochondrial sequence to each other, but differed from the *H. m. morphnoides* sequences. Parsimony and Bayesian analyses show that *H. m. weiskei* is most closely related to *H. pennatus* with weak support (sequence identity for cyt-b = 97.8%, for ND2 = 98.1%; PP =0.63, bs=95). *H. m. morphnoides* and *H. m. weiskei* are slightly more divergent: sequence identity for cyt-b is 97.3% and for ND2 is 94.2%. Bayesian posterior probability is 1.00 and the bootstrap support is 100 for the node separating *H. m. morphnoides* from *H. m. weiskei* and *H. pennatus*.

Sea eagles (Haliaeetinae).—The sea eagles form a well-supported monophyletic group in the mt dataset consisting of two genera: *Haliaeetus* and *Ichthyophaga*. In this analysis

the genus *Haliaeetus* is paraphyletic when the two *Ichthyophaga* species are included. Forcing monophyly of the genus *Haliaeetus*, requires 7 additional steps.

Analyses of the nuc + mt data support a sister relationship between the sea eagles and kites in the genus *Haliastur* (PP = 1.00, bs=90), however a sister relationship between the Milvinae and Haliaeetinae was not recovered with the mt dataset

Harpy eagles (Harpiinae*)*.—Three of four proposed harpy eagles form a clade with high support (PP = 1.00, 1.00, bs=95, 97): *Harpia harpyja*, *Morphnus guianensis* and *Harpyopsis novaeguineae*. These 3 species are highly similar in sequence (~91% identical). A fourth species typically included in the Harpy eagle group, the Philippine eagle (*Pithecophaga jefferyi),* is placed sister to a clade of snake eagles (Circaetinae) which is distant from and earlier diverging than the three species found here to belong to the Harpiinae. The support values for these relationships are high in all analyses. We are unaware of any other analysis suggesting a relationship between the Philippine eagle and the Circaetinae. Given this exceptional result in our dataset, we took extra measures to confirm the validity of the sequence and its phylogenetic placement. We sequenced two individual Philippine eagles for all three genes and portions of all three genes for a third individual. All sequences obtained were identical for the three individuals, and uniquely different from other species in the dataset. This novel finding is also corroborated by the distribution of indels noted previously. In particular, the Philippine eagle lacks an eight base deletion in BF-I7 that is shared by the three other traditional members of the harpy eagle group (lack of monophyly for the harpy eagle group species is consistent whether gaps are counted as missing data or as a 5^{th} base state in MP analyses). Forcing

monophyly of the traditional harpy eagle group (4 members) would require an additional 51 parsimony steps.

It was proposed that the two species in the genus *Harpyhaliaetus* are members of the harpy eagle group or are closely related (Brown, 1970). In our analyses *Harpyhaliaetus solitarius* and *Harpyhaliaetus coronatus* are placed within the Buteoninae and, more specifically, within a clade of two *Buteogallus* species. Neither *Harpyhaliaetus* species shares the eight base deletion in BF-I7 found in three members of the harpy eagle group. Forcing monophyly of the harpy eagle group including all six potential members increases the tree length by 292 steps.

Circaetinae (snake eagles).—All of the snake eagles form a monophyletic group sister to the Old World vulture group Aegypiinae, except the Madagascar serpent-eagle (*Eutriorchis astur*) which is placed within the Gypaetinae. Forcing monophyly of all snake eagles requires an additional 168 parsimony steps.

The genus *Circaetus* is not monophyletic in these analyses when the West African serpent-eagle (*Dryotriorchis spectabilis*) is included. An additional 7 parsimony steps are needed to force monophyly of the genus *Circaetus*.

The Bayesian posterior probability for the node uniting the Philippine eagle and the African snake eagles (rather than the non-African snake eagles) was high (PP=0.84, 1.00). The MP analyses recovered a sister relationship between the Philippine eagle and the bateleur albeit with low support (bs=52, 55).

Old World vultures (Aegypiinae and Gypaetinae).—The Old World vultures also do not form a monophyletic group, but form two separate clades in the analyses (Aegypiinae and Gypaetinae). Each of the Gypaetinae species (*Gypohierax angolensis, Eutriorchis astur, Neophron percnopterus* and *Gypaetus barbatus*) are highly divergent from each other genetically (sequence identities ~87%) but are more closely related to each other than to other Accipitridae species (PP = 0.80). Here we also find that the Madagascar snake eagle is a member of the Gypaetinae, a relationship not proposed before. All remaining Old World vultures form a separate clade (Aegypinae) with a close relationship to other snake eagles (Circaetinae). The relationships within this clade of vultures, the Aegypiinae, are highly concordant in all analyses except the position of *Necrosyrtes monachus*. This species is more closely related to, although highly divergent from, the species of the genus *Gyps* than to the other four monotypic Aegypiinae genera with high support in the Bayesian analyses (PP = 1.00, 0.99), but is sister to the other Aegypiinae taxa in the parsimony analyses (bs = 0.54, unresolved in the nuc + mt dataset). Necrosyrtes also lacks the ND2 insertion of two bases that all other Aegypiinae species share.

Discussion

We have presented data from both mitochondrial and nuclear sequences for approximately 50% of the recognized species in the Accipitridae, focusing on groups commonly known as eagles and Old World vultures with nearly complete species representation. This is the most complete systematic treatment of the Accipitridae family to date based on molecular data. We found strong evidence for non-monophyly of some existing genera and subfamilies. Although Accipitridae subfamilies are infrequently used

in recent classifications we agree with Brown (1976) that subfamilies are useful in clarifying relationships among these diverse birds. Designation of subfamilies is not our primary goal; however, we use and reconfigure the twelve existing subfamilies and recognize two new subfamilies in an effort to make the evolutionary history of the Accipitridae more easily understood (Table 1). Our analyses included representatives of all 14 primary Accipitridae clades that have been recognized by previous researchers. In the following section we discuss the taxonomic history of the focal subfamilies and several examples of convergences, generally involving traits related to feeding habits, revealed by findings of non-monophyly for traditional taxa.

Booted eagles (Aquilinae).—We found good support for monophyly of the booted eagles (Figs. 1 and 2), corroborating earlier morphological assessments. Proposed phylogenetic relationships and taxonomy within the booted eagles, however, have a long history of confusion and revision among authors. Our analyses confirm that the three main genera (*Aquila*, *Hieraaetus* and *Spizaetus*) are not monophyletic, a result suspected by many morphologists but that has been difficult to resolve with morphological traits.

Our data support an early diverging clade of Asian hawk-eagles (*Spizaetus* species) separate from the New World hawk-eagles (*Spizaetus spp*., *Oroaetus sp.* and *Spizastur sp.*). Brown and Amadon (1968) recognized that the Asian *Spizaetus* species are more similar to each other morphologically than to the other *Spizaetus* species, but did not separate the genus accordingly. Within the Asian hawk-eagle clade we find support for sister relationships between *S. cirrhatus* and *S. lanceolatus*, and *S. alboniger*

and *S. nipalensis*. Within each pairing, the two species have largely overlapping ranges and are similar morphologically (Ferguson-Lees, Christie, 2001).

The New World hawk-eagles comprise three genera (including *Spizaetus*) and are all each others closest relatives, forming a separate clade within the booted eagles that is not sister to the Old World hawk-eagles. These species have largely overlapping ranges within the New World but are found in vastly different habitat types ranging from open areas (*Spizaetus tyrannus*) to heavily forested regions at higher altitude (*Oroaetus isidori*).

Three Old World species each branch off separately within the Aquilinae and are shown not to have any close relationships with other species: the crowned hawk-eagle (*Stephanoaetus coronatus*), the rufous-bellied eagle (*Hieraaetus kienerii*), and the Martial eagle (*Polemaetus bellicosus*). Both the crowned hawk-eagle and the martial eagle have been placed in monotypic genera because of their divergent morphology. Genetically these birds are also highly divergent from other booted eagles in our dataset. Recently the rufous-bellied eagle was recognized as a member of the genus *Hieraaetus* (Dickinson, 2003), although monophyly of the genus *Hieraaetus* has been questioned (Brown, Amadon, 1968). The rufous-bellied eagle is a morphologically specialized bird having long toes, a crest, and adult plumage that is dissimilar from the other booted eagles. Here it is shown that it is genetically distant from other extant booted eagles, and phylogenetically distinct from its current congeners in *Hieraaetus*.

The well-supported clade including the Asian black eagle (*Ictinaetus malayensis*), the long-crested eagle (*Lophaetus occipitalis*) of Africa and two species in the genus *Aquila* has not been proposed before. The species of these two monotypic genera are

highly unique in morphology. The long-crested eagle is an African woodland species found in moist savannahs and riverine strips feeding on rodents, while the Asian black eagle is a resident of mountain woodlands with morphological traits that accompany its feeding specialization on bird's eggs and young. The other two species in this clade, the lesser spotted eagle (*Aquila pomarina*) and the greater spotted eagle (*Aquila clanga*) are difficult to separate morphologically and hybrids of the two species have been documented (Vali, Lohmus, 2004). The two specimens we sampled were significantly different genetically, but clearly more closely related to each other than any of the other accipitrid taxa in the study.

The next three diverging Aquilinae clades include species from the genera *Hieraaetus* and *Aquila*, and one species currently in the genus *Spizaetus*. Most of these species have been recognized as members of different genera in the past. Brown and Amadon (1968) separate *Hieraaetus* species from those in the genus *Aquila* by morphological traits. *Hieraaetus* species appear generally smaller than eagles in the genus *Aquila*, with a smaller bill, longer and more slender legs and deeper emargination on primaries; however, these characters do not hold for all species in these genera. In our analyses we find members of these two genera intermixed with each other and with Cassin's hawk-eagle (*Spizaetus africanus*) such that, again, none of these genera are monophyletic. One of these clades includes six closely related species: *A. chrysaetos, Spizaetus africanus, H. fasciatus, A. verreauxii, A. audax,* and *A. gurneyi*. A close relationship among *A. gurneyi, A. chrysaetos, A. audax* and *A. verreauxii* has been proposed based on morphological data (Brown and Amadon, 1968). Cassin's hawk-eagle is morphologically divergent from these four *Aquila* species so it was not previously

included in that group. This species has been placed in the genus *Hieraaetus* (Thiollay, 1994) and a monotypic genus (Cassin, 1865), but has not been a member of the genus *Aquila*. The remaining two species (*H. fasciatus* and *H. spilogaster*) in this clade have sometimes been considered as conspecific subspecies (see below).

Three species currently placed in the genus *Aquila* (*A. nipalensis, A. rapax and A. heliaca*) form a monophyletic group whereas seven other *Aquila* species are separated from these three and, variously, from each other (Fig. 1). The close relationship of these species relative to each other rather than the remaining booted eagle species is clear from morphological data, our analysis and some previously published genetic data (Vali, 2002). The placement of these three species separate from the other seven congeners in this study supports the need for taxonomic revision of the genus *Aquila*, so that it designates a monophyletic group.

The final clade of booted eagles in our analyses includes four currently recognized species with wide distributions and habitats: *Aquila wahlbergi, Hieraaetus ayresii, H. morphnoides* and *H. pennatus*. All but one of these species is in the genus *Hieraaetus*. This outlying species, Wahlberg's eagle (*A. wahlbergi*) is an Afrotropical species of wooded savannah or bushveld. It has been placed in the genus *Hieraaetus* as well as the genus *Spizaetus* before.

Two booted eagle clades identified in our analyses correspond closely to the geographical distribution of species: Indomalayan hawk-eagles of the genus *Spizaetus* form a clade separate from the New World hawk-eagles (*Spizaetus, Spizastur* and *Oroaetus*). The remaining booted eagle clades show evidence of only one other

(apparent) colonization of booted eagles in the New World, which is by the Golden eagle (*Aquila chrysaetos*), a species that is found in both the Old and New Worlds.

Aquilinae Subspecies.—While our study has focused on recognized eagle species, we realize that some taxa currently classified as subspecies might be better elevated to species. The results of such analyses could have important implications for conservation, as many Accipitridae species are declining or endangered.

In the first case we sampled multiple representatives of the two known subspecies of *Hieraaetus morphnoides* (*H. m. morphnoides* and *H. m. weiskei*) which do not overlap in range. *H. m. morphnoides* is found only in Australia whereas *H. m. weiskei* is found only in New Guinea. Furthermore, *H. m. weiskei* is both smaller in size and darker in color than *H. m. morphnoides*. Although Brown and Amadon (1968) reported the differences between these two subspecies and a close relationship between the two species *H. pennatus* and *H. morphnoides* they maintained subspecies status for these birds. Other authors have elevated the two to species status (in Brown, Amadon, 1968). While sister relationships among these three *Hieraaetus* taxa is not entirely resolved here, the amount of sequence variation between *H. m. morphnoides* and *H. morphnoides weiskei* is as much as is found between species in this analysis. This result is based on the sampling of multiple individuals of each species/subspecies in our analysis and a previous study (Bunce *et al.*, 2005) and supports the phylogenetic distinctiveness and recognition of *H. m. weiskei* and *H. m. morphnoides* as separate species (*H. weiskei* and *H. morphnoides*, respectively).

In the second case we sampled multiple individuals of *Hieraaetus fasciatus*. The distribution of *H. fasciatus* is disjunct in that birds that reside year-round in southern Africa are separated from migratory birds found in Europe, northern Africa, Asia and India. Some of the more northern birds migrate to spend the winter in southern Africa, but do not remain to breed. South African birds are also smaller in size with recognizable plumage differences. The taxonomy of *H. fasciatus* has long been debated. Brown and Amadon (1968) recognized one species *H. fasciatus* with two subspecies noting the distinct appearance but similar habits of the South African representatives (*H. f. spilogaster*). Two *H. f. spilogaster* individuals that are residents of the countries of South Africa and a third from Zimbabwe form a distinct lineage separate from the *H. f. fasciatus* individuals in our analyses with high Bayesian posterior probability. Genetic distances between the *H. f. spilogaster* individuals and the *H. f. fasciatus* individuals are slightly greater than that of other sister species pairings in booted eagles, such as *H. morphnoides* and *H. pennatus*, and *Aquila audax* and *A. gurneyi* (95%, 98%, 97% sequence similarity respectively). Our findings suggest that further study with greater sampling of individuals is warranted in order to determine if *H. f. spilogaster* should be elevated to species status (*H. spilogaster*) and, if so, where the limits of its distribution lie.

Sea eagles (Haliaeetinae).—Sea eagles have long been considered to be a monophyletic group with a close relationship to the Milvinae kites. This relationship is largely based on the shared trait of fusion of the second and third phalanges found in all sea eagles and the Milvinae kites (Holdaway, 1994), but not in other accipitrid taxa. Previous molecular

studies supported monophyly of the sea eagles in the genus *Haliaeetus* (Seibold, Helbig, 1996), and indicated a close relationship between single species representatives of the two groups (Mindell et al. 1997). Here, with more comprehensive sampling, we support monophyly of the subfamily Haliaeetinae, but not of the genus *Haliaeetus* when the other sea eagle genus, *Ichthyophaga*, is included in analyses. We also support a sister relationship between the Milvinae kites (species in the genera *Milvus* and *Haliastur* only) and the sea eagles with the nuc + mt dataset.

The monotypic palmnut vulture (*Gypohierax angolensis*) is one of the few frugivorous Accipitridae species, eating palm fruits, and occasionally fish, crabs, snails and other small animals. Behavioral and morphological traits, such as rounding of the underside of the talons, suggest a relationship between the sea eagles and the palmnut vulture. Brown and Amadon (1968), and Jollie (1977) note that it resembles the Egyptian vulture (*Neophron*). In a phylogenetic analysis of osteological characters, Holdaway (1994) found support for a monophyletic group of vultures with the palmnut vulture as the earliest diverging lineage. Here we present the first molecular evidence that the palmnut vulture is an early diverging Old World vulture species more closely related to the lammergeyer (*Gypaetus barbatus*), the Madagascar snake eagle and the Egyptian vulture. Thus, the similarities between the sea eagles and the palmnut vulture are clearly convergent in nature.

The sea eagles of the genus *Haliaeetus* are neatly divided by a split between species with northern distributions (*H. albicilla, H. leucocephalus* and *H. pelagicus*) and species of tropical distributions (*H. vocifer, H. vociferoides, H. leucogaster* and *H. sanfordi*). *H. leucoryphus* is a year-round resident of the tropics, however this species

also breeds in the northern temperate region. Here, we find that *H. leucoryphus* clusters with the northern species. These results are similar to those found by Seibold and Helbig (1996). We also included three taxa not represented in previous studies: *Ichthyophaga ichthyaetus, I. humilis* and *H. vociferoides*. These three species further support the tropical—temperate split, as all of these species have tropical distributions and are found to be members of the tropical clade. *H. vociferoides*, the Madagascar sea eagle and *H. vocifer*, the African sea eagle are sister species, a relationship also supported by their unique reddish plumage and complex, melodious vocalizations.

Harpy eagles (Harpiinae).—The members of the harpy eagle group (as defined by Brown and Amadon, 1968) are easily distinguished from other Accipitridae eagles by traits such as their extremely large size, with female wing-spans ranging from 1.76 to 2.01 meters in length and female body weights ranging from six to nine kilograms in *Pithecophaga* and *Harpia* (Ferguson-Lees, Christie, 2001). All of the traditional harpy eagle group members live in primary tropical forest, preying on medium-sized mammals (e.g. monkeys, sloths, tree kangaroos). There are two Old World species, the Philippine eagle (*Pithecophaga jefferyi*) and the New Guinea harpy eagle (*Harpyopsis novaeguineae*), and two New World harpy eagles, the harpy eagle (*Harpia harpyja*) and the crested eagle (*Morphnus guianensis*). Brown and Amadon (1968) suggested that specialization in tropical forests and on a diet of mammals may have led to convergent characters such that the Old World species are not closely related to the New World species. Our data partially agree with this hypothesis as we found strong support for one of the Old World species, the Philippine eagle, being more closely related to the snake

eagles (Circaetinae) than to the three others species in the traditional harpy eagle group. Therefore, we do not include the Philippine eagle in the Harpiinae.

The two *Harpyhaliaetus* species are not members of the Harpy eagle group, but are more closely related to the two *Buteogallus* species, a relationship proposed by Brown and Amadon (1968). The genus *Buteogallus* is paraphyletic in our analyses when the *Harpyhaliaetus* species are included.

Snake eagles (Circaetinae).—Genetic data has previously been published for only two of the snake eagles, and morphologists have had difficulty identifying species that are closely related to the snake eagle group. Here we present strong evidence that, when the Madagascar serpent-eagle is excluded, the snake eagles form a monophyletic subfamily (Circaetinae) that is most closely related to some Old World vultures (Aegypiinae) and the Philippine eagle than to other Accipitridae. We are the first to propose that the Madagascar serpent-eagle (*Eutriorchis astur*) is not a member of the clade including the other snake eagles. For instance, Brown and Amadon (1968) suggested that *Eutriorchis* and *Dryotriorchis* should be united in one genus, or, based on the shape of the crown feathers, that the Madagascar serpent-eagle is more closely related to the *Spilornis* species. In our analyses the Madagascar serpent-eagle clusters with three Old World vultures in the subfamily Gypaetinae.

Another surprising finding is the placement of the Philippine eagle (*Pithecophaga jefferyi*) within the snake eagle clade (see section 4.3).

The *Spilornis* species are extremely rare and generally island endemics in the Indomalayan region, a region of high species loss and conservation importance (Collar *et*

al., 2001; Mooers, Atkins, 2003). Jepson et al. (2001) suggest that Indonesia's lowland forests will entirely vanish by 2006 and the outlook for Malaysian forests is similarly poor. This situation highlights the importance of assessing the phylogenetic history and genetic distinctiveness of the Indonesian *Spilornis* species. Here we included four *Spilornis* species, Brown and Amadon (1968) proposed five, Sibley and Monroe (1990) recognized six and Ferguson-Lees et al (2001) identified 13 *Spilornis* species. We suggest relationships of all of the snake eagle species and subspecies should be further explored with increased sampling to inform attempts to conserve these unique and relatively little known taxa.

Snake eagles are found only in the Old World and mainly in the Indomalayan and the Afrotropical regions. One species (*Circaetus gallicus*) is found in the western part of the Palearctic region. The deepest split within the snake eagles corresponds largely to their geographic distribution where Indomalayan species form a clade separate from the Afrotropical species. Only the Philippine eagle does not follow this pattern as it is more closely related to the African snake eagles. The West African serpent-eagle (*Dryotriorchis spectabilis*) falls within a clade of species in the genus *Circaetus*, suggesting that the taxonomy of these two genera should be revised.

Old World vultures (Aegypiinae and Gypaetinae).—Old World vultures have been proposed to be monophyletic (Brown, Amadon, 1968; Thiollay, 1994) or polyphyletic with *Gyophierax, Neophron* and *Gypaetus* forming one or more groups separate from the others (Jollie, 1977b; Mundy *et al.*, 1992; Seibold, Helbig, 1995). Sister groups have not been identified for the Old World vultures, although the palmnut vulture (see 4.2.1) was

proposed to represent the "transition" from vultures to sea eagles (Brown, Amadon, 1968). Here we find clear support for two separate subfamilies of different evolutionary origin. The Gypaetinae is the earlier diverging group, and its constituent taxa (including *Gypohierax*, *Gypaetus*, *Eutriorchis* and *Neophron*) are relatively divergent genetically as well as morphologically. The remaining vultures form a monophyletic group, the Aegypiinae, sister to the Circaetinae snake eagles. The phylogenetic position of *Necrosyrtes* within the Aegypiinae remains uncertain.

Kites (Milvinae, Perninae, Elaninae.—Friedmann (1950a) described three kite subfamilies (Milvinae, Perninae and Elaninae) without identifying sister relationships among them, and considered them as early diverging Accipitridae taxa. Brown and Amadon (1968) considered kites to be the most "primitive" Accipitridae group due to their specialization on insects (e.g. bee and wasp larvae) or snails. Here we provide evidence of at least four distinct clades, including the three traditional kite subfamilies with some novel hypotheses of relationships, and two non-sister lineages within the Buteoninae. The non-sister relationship for *Ictinia* and *Rostrhamus* requires further analysis as nodal support values are relatively low and taxonomic representation of kites and Buteoninae is limited.

Convergent evolution in Polyboroides and Geranospiza .—The gymnogene (*Polyboroides typus*) and the crane hawk (*Geranospiza caerulescens*) are specialized birds that have developed a series of morphological characteristics related to capturing birds in cavity nests or other small animals in holes or crevices. These traits include an

extended circular range of motion for the tarsus, a short outer toe and a relatively weak bill. Based on these traits a close relationship between the two species has been proposed (Friedmann, 1950a). Some morphological differences between these two species, including differences in extent of tarsus rotation, suggest that two different evolutionary paths led to the traits allowing exploitation of cavity nesting species (Burton, 1978). These two species are not closely related in our analyses, denoting a clear example of convergent evolution in specialized morphology in the Accipitridae.

Conclusions

This study takes a large step toward resolving the uncertain relationships among birds in the Accipitridae. Our analyses include over 3,000 bases of nuclear and mitochondrial DNA and a sampling of almost half of the known Accipitridae species, with nearly complete species sampling for eagles and Old World vultures. We find support for a set of phylogenetic relationships among Accipitridae taxa that differ from previous hypotheses based on morphological data. Fourteen subfamilies, of which two are new, are discussed here in order to represent the diversity and evolutionary history of Accipitridae taxa in a manner reflecting our findings. If taxonomy is to reflect phylogeny, revisions are warranted within the booted eagle (Aquilinae), snake eagle (Circaetinae) sea eagle (Haliaeetinae) and harpy eagle (Harpiinae) groups. We report significant genetic differentiation among several sets of subspecies investigated, suggesting that further analyses, particularly of booted eagles, should include multiple samples of species across their ranges or representing described subspecies. The rarity and threatened status of many of the Accipitridae species make such investigations of imminent importance to conservation.

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Author note

After this article was accepted for publication a treatment of a subset of booted eagles (Aquilinae) using cyt-b and additional nuclear sequences was published by Helbig et al. (2005). The findings of Helbig et al. are concordant with our study, as are the cyt-b sequences with the notable exception of *Aquila pomarina*. While both analyses place *A. pomarina* as sister to *A. clanga* with high support, the cyt-b sequences are relatively dissimilar (87.4% similarity index). *Aquila pomarina* has a disjunct population distribution with separate Indian and European populations; the Indian population is morphologically distinct and denoted by the subspecies *A. p. hastata*. The specimen used in our study is from the Indian population/subspecies, while Helbig et al. used a specimen of European origin. This large sequence divergence between two specimens from separate populations of the same species suggests that further study of the populations of this species is warranted. The placement of the crowned hawk-eagle (*Stephanoaetus coronatus*) also differs between the two studies. We find the crowned hawk-eagle to be the first diverging species after the Old and New World hawk-eagles, while Helbig et al. support a sister relationship between Old World hawk-eagles and the crowned hawk-eagle. This difference is likely a result of the larger and slightly different taxon set used in our study, as we do not find a sister relationship between the crowned hawk-eagle and the Old World hawk-eagles even when we analyze our cyt b sequences separately from the other sequences in our study.

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Chapter 3

Molecular phylogenetics of the buteonine birds of prey (Aves: Accipitridae)

The Buteoninae subfamily of hawk, hawk-like and kite species forms one of the largest groups in the avian Accipitridae family including 24 sub-buteo species (Amadon, 1982a) two kite genera (Lerner, Mindell, 2005) and 25-28 *Buteo* species (Dickinson, 2003; Ferguson-Lees, Christie, 2001). They are of particular interest as eleven species are of conservation concern (Baillie *et al.*, 2004) with one critically endangered (*Buteo ridgwayi*) and two endangered species (*Leucopternis occidentalis* and *Harpyhaliaetus coronatus*). Buteoninae has also included the sea and booted eagles (Grossman, Hamlet, 1964) or the sea, booted and harpy eagles (Friedmann, 1950b). Our recent molecular analysis, however, showed that the sea, booted and harpy eagles form monophyletic groups separate from a clade of ten sub-buteos, two kites and three *Buteo* species (Lerner, Mindell, 2005). Therefore, we do not consider any of the eagle groups as members of Buteoninae. For purposes of this study, we consider Buteoninae to be comprised of the genus *Buteo* and the nine sub-buteo and two kite genera all previously proposed as or found to be close relatives of *Buteo*: New World hawks *Leucopternis*, *Buteogallus* (including *Heterospizias*), *Harpyhaliaetus*, *Busarellus*, *Parabuteo*, *Geranoaetus*, *Asturina* (now within *Buteo*) and *Geranospiza*; Old World hawks *Kaupifalco* and *Butastur* (Amadon, 1982a); and, kites *Ictinia* and *Rostrhamus* (Lerner, Mindell, 2005).

While polyphyly of the sub-buteo group with respect to *Buteo* has long been suspected, only recently has it been shown that the genera *Buteo*, *Leucopternis* and *Buteogallus* are not monophyletic with respect to each other (do Amaral *et al.*, 2006; Lerner, Mindell, 2005; Riesing *et al.*, 2003b). The full extent of polyphyletic relationships in Buteoninae is not known since not all nominal species and subspecies have been included in a single analysis. Further, previous analyses have not tested Buteoninae phylogenetic relationships in context of the other major accipitrid clades. In particular, the placement of the Lizard Buzzard (*Kaupifalco monogrammicus*), Black-collared Hawk (*Busarellus nigricollis*) and genus *Butastur* remains to be assessed with molecular data in the broader context of the Accipitridae. The three species of *Butastur* have not previously been included in peer-reviewed molecular datasets. Neither *Kaupifalco* nor *Busarellus* formed close sister relationships with three other sub-buteo genera, 25 *Buteo* species, a booted eagle and an accipiter in a study by Riesing et al. (2003b) using mitochondrial NADH dehydrogenase subunit 6 (ND6) and pseudo-control region. With a phylogeny generated from 191 osteological characters for 44 Accipitrid taxa, Holdaway (1994) did not find a close relationship between *Kaupifalco* and any other accipitrid species. In the same study, *Busarellus* was sister to booted eagles *Hieraaetus* and *Polemaetus* although nodal support values were not presented for the phylogeny.

Species status has been questioned for taxa in the sub-buteonine genera *Buteogallus* (*B. anthracinus*, *B. subtilis* and *B. aequinoctialis*) and *Leucopternis* (*L. schistaceus* and *L. plumbeus*; *L. kuhli* and *L. melanops*; and, *L. albicollis*, *L. occidentalis* and *L. polionotus*) where widespread taxa occupying similar niches have been divided

into multiple subspecies or separate species without conclusive evidence one way or the other (Amadon, 1982a). A recent mitochondrial phylogeny found sister relationships for *L. kuhli* and *L. melanops* and *L. albicollis*, *L. occidentalis* and *L. polionotus* but not for *L. schistaceus* and *L. plumbeus* (do Amaral *et al.*, 2006). Still, the other questioned *Leucopternis* and *Buteogallus* groups have not been tested with molecular data and further testing of most of these groups is needed to evaluate current taxonomy.

A comprehensive analysis of the phylogenetic relationships among proposed buteonine genera and species is needed to address remaining questions about the group's evolutionary history. With complete taxonomic representation of genera and nearly all nominal species and sub-species of sub-buteos, we address the following questions: (1) are *Kaupifalco*, *Busarellus* and *Butastur* closely related to other proposed buteonines? (2) what are the sister relationships among Buteoninae genera? (3) to what extent are the genera polyphyletic? (4) is there evidence of genetic divergence and reciprocal monophyly to support current taxonomy for species and subspecies of *Buteogallus* and *Leucopternis*?

Methods

We sampled at least one individual of each nominal genus, species and nearly all subspecies of sub-buteos. Our final sampling included 107 individuals representing 45 out of 55 buteonine species, 26 out of 176 non-Buteonine accipitrid species and 2 non-accipitrid outgroup species (Table 1, Dickinson, 2003; Ferguson-Lees, Christie, 2001). To test monophyly of Buteoninae we included representatives of each recognized Accipitridae subfamily/clade. We also included multiple representatives of *Circus*,

Table 5. Sample information.

Genus species[a]	Voucher or Tissue ID[f], Tissue type[c]	Dataset			Locality
		mt[d]	mt + bf	ND6[e]	
Accipiter bicolor guttifer[b]	LSUMZ B18875, T	√	√		Santa Cruz Dept., Bolivia
Accipiter cooperii[b]	KUNH 1757, T	√	√		Unknown, U.S.A.
Accipiter gentilis atricapillus	UMMZ 233684, T	√			Michigan, U.S.A.
Accipiter gularis	LSUMZ 16971, T	√			Saitama Prefecture, Japan
Accipiter c. cirrocephalus	AMS O.65038, T	√	√		New South Whales, Australia
Accipiter n. nisus	KUNH 4501, T	√			Entracque, Italy
Accipiter r. rufiventris	PBC 19, T, P	√	√		South Africa
Aegypius monachus[b]	DZ 1903, B	√	√		Captive, Unknown
Asturina nitida/Buteo nitidus pallida	LSUMZ B9624, T	√	√	√	Nicolás Suarez, Bolivia
Busarellus nigricollis leucocephalus	UMMZ 105267, M	√		√	Paraguay
Butastur indicus	UMMZ 65937, M	√			Ishigaki, Japan
Butastur rufipennis	UMMZ A1290, T	√	√	√	Gambia
Butastur teesa	UMMZ 209040, M	√			Kampur, India
Buteo albicaudatus hypospodius	MSB 20414, T	√	√	√	Texas, U.S.A.
Buteo albigula	LSUMZ 31984, T	√	√	√	Quebrada Lanchal, Peru
Buteo jamaicensis[b]	UMMZ T-2797, T	√	√	√	North America
Buteo lagopus sanctijohannis	KUNHM 3450, T	√	√		Kansas, U.S.A.
Buteo/Percnohierax leucorrhous	ZMUC P526 (113928), D	√	√	√	Cotopaxi, Ecuador
Buteo lineatus	LSUMZ B1344, T	√	√		unkown
Buteo/Rupornis magnirostris occiduus[b]	LSUMZ B2862, T	√	√	√	Loreto Dept, Peru
Buteo oreophilus trizonotatus	WOB 17, B, P	√	√		South Africa
Buteo p. platypterus	UMMZ DM36, T	√	√	√	Michigan, U.S.A.
Buteo poecilochrous	HUA 8, B, P	√	√	√	Peru

Buteo p. polyosoma	LSUMZ B5135, T	√	√	√	Las Pampas, Peru
Buteo regalis	KUNHM 1767, T	√	√	√	Kansas, U.S.A.
Buteo rufinus	UMMZ DM54, T	√	√		unknown
Buteo rufofuscus	JBZ 5, B	√	√	√	South Africa
Buteo swainsoni	UMMZ DM11, T	√	√	√	unknown
Buteogallus aequinoctialis	UMMZ 116637, M	√			Matapica, Surinam
Buteogallus a. anthracinus[b]	LSUMZ B28575, T	√	√		Fort Sherman, Panama
Buteogallus meridionalis	UMMZ 155624, M	√	√	√	El Pao, Venezuela
Buteogallus subtilis bangsi	UMMZ 132087, M	√	√		Pigres, Costa Rica
Buteogallus urubitinga ridgwayi	UMMZ 132082, T	√	√	√	Catalina, Costa Rica
Busarellus nigricollis leucocephalus	UMMZ 105267, M	√	√	√	Riacho Negro, Paraguay
Chondrohierax uncinatus[b]	TPF 147, B, P	√	√		Grenada
Circaetus cinereus[b]	PNZ 8, B, P	√	√		South Africa
Circaetus gallicus[b]	TAU 363, T	√	√		Unknown
Circus aeruginosus [b]	TAU 353, T	√	√		Unknown
Circus ranivorus[b]	PBC 6, B, P	√	√		South Africa
Elanus leucurus majusculus	LSUMZ 24997, T	√	√		Texas, U.S.A.
Geranoaetus/Buteo melanoleucus australis[b]	HUA 3, B, P	√	√	√	Peru
Geranospiza caerulescens	LSUMZ B4226, T	√	√		Peru
Geranospiza caerulescens flexipes[b]	KUNHM 3110, T	√	√		Paraguay
Haliaeetus leucocephalus[b]	UMRC N42, T	√	√		North America
Haliastur sphenurus[b]	SAM NTMT651, ABTC-27746, T	√	√		Northern Territory, Australia
Hamirostra melanosternonb	AMS 1, F	√	√		Australia
Harpyhaliaetus coronatus[b]	UMMZ 101669, M	√			Amambay, Paraguay
Harpyhaliaetus s. solitarius[b]	HUA 18, B	√	√	√	Peru
Ictinia plumbea	KUNHM 2900, T	√	√		Paraguay
Ictinia mississippiensis	KUNHM B1581, T	√			Lousiana, USA
Kaupifalco monogrammicus	UMMZ 214672, M	√			Mozambique

meridionalis					
Leptodon cayanensis[b]	KUNHM 139, T	√	√		Paraguay
Leucopternis a. albicollis	ZMUC P1517 (114919), D	√	√	√	Tigre Playa Sucumbios, Ecuador
Leucopternis a. albicollis	HUA 10, B, P	√	√	√	Selva Central, Peru
Leucopternis. a. albicollis	HUA 11, B, P	√	√	√	Selva Central, Peru
Leucopternis. a. albicollis	HUA 12, B, P	√	√	√	El Huayco, Peru
Leucopternis a. albicollis	UMMZ 117773, M	√			Surinam
Leucopternis albicollis costaricensis	LSUMZ B2312, T	√	√	√	Panama
Leucopternis albicollis costaricensis	TPF WHH-024, B	√	√	√	Panama
Leucopternis albicollis costaricensis	UMMZ 56218, M	√			Barro Colorado Island, Panama
Leucopternis albicollis costaricensis	UMMZ 85741, M	√			Nicaragua
Leucopternis albicollis costaricensis	UMMZ 199396, M	√			Honduras
Leucopternis albicollis ghiesbreghti	TPF, LM-0, B	√	√	√	Tikal National Park, Guatemala
Leucopternis. albicollis ghiesbreghti	TPF, LM-1, B	√	√	√	Naranjol, Guatemala
Leucopternis albicollis ghiesbreghti	TPF, LM-2, B	√	√	√	Yucatan Peninsula
Leucopternis. albicollis ghiesbreghti	UMMZ 210554, M	√			Oaxaca, Mexico
Leucopternis albicollis ghiesbreghti	UMMZ 94013, M	√			Chiapas, Mexico
Leucopternis albicollis williaminae	USNM 372349, M	√			Cesar, Colombia
Leucopternis albicollis williaminae (TYPE)	ANSP 160392, M	√			Bolivar, Colombia
Leucopternis kuhli[b]	LSUMZ B4598, T	√	√	√	South Rio Amazonas, Peru
Leucopternis kuhli	FMNH 101120, M	√			Brazil
Leucopternis kuhli	FMNH 297880, M	√			Peru
Leucopternis kuhli	USNH 512908, M	√			Para, Brazil
Leucopternis lacernulatus	AMNH 317243, M	√			Espirito Santo, Brazil
Leucopternis melanops	LSUMZ B4493, T	√	√	√	Lower Rio Napo, Peru
Leucopternis melanops[b]	LSUMZ B7167, T	√	√	√	Peru
Leucopternis melanops	FMNH 260137, M	√		√	Surinam
Leucopternis melanops	AMNH 471056, M	√			Caura, Venezuela
Leucopternis occidentalis	UMMZ DM BE5, T	√	√	√	unknown

Leucopternis occidentalis	LSUMZ B7805, T	√	√	√	Ecuador
Leucopternis occidentalis	LSUMZ B7890, T	√	√	√	Ecuador
Leucopternis occidentalis[b]	ZMUC P1319 (114721), D	√	√	√	Esmeraldas, Ecuador
Leucopternis plumbeus	BMNH 1939.12.9.295, M	√			Perme
Leucopternis plumbeus	BMNH 1955.6.n.20.2453, M	√	√		Ecuador
Leucopternis polionotus	BMNH 1895.4.1.510, M	√	√		Rio de Janeiro, Brazil
Leucopternis polionotus	USNM 264120, M	√			Santa Catharina, Brazil
Leucopternis princeps zimmeri	LSUMZ B11751, T	√	√	√	Ecuador
Leucopternis p. princeps	AMNH 389182, M	√			Turrialba, Costa Rica
Leucopternis schistaceus	LSUMZ B4946, T	√	√	√	S Rio Amazonas, Peru
Leucopternis schistaceus	FMNH 217636, M	√			Bolivia
Leucopternis semiplumbeus	LSUMZ B2291, T	√	√	√	Panama
Leucopternis semiplumbeus	LSUMZ B2326, T	√	√	√	Panama
Leucopternis semiplumbeus	UMMZ DM35, T	√			Unknown
Lophoictinia isura[b]	AMS 0.7591, F	√			Australia
Melierax canorus	WOB 7, B, P	√	√		South Africa
Melierax poliopterus	TPF MB-15, F, P	√			Unknown
Micronisus g. gabar[b]	UMMZ A765, T	√	√		Zimbabwe
Oroaetus isidori[b]	HUA 23, B, P	√	√		Peru
Parabuteo unicinctus harrisi[b]	UMMZ DM40, T	√	√	√	Arizona, U.S.A.
Rostrhamus s. sociablis[b]	KUNHM 5852, T	√	√		Guyana
Spizaetus ornatus vicarious[b]	LSUMZ B2267, T	√	√		Darien Province, Panama
Torgos tracheliotus[b]	UMMZ 234705, T	√	√		South Africa
Urotriorchis macrourus	FMNH 204470, M	√			Centre Sud, Cameroon
Sagittarius serpentarius[b]	JBZ 12, B, P	√	√		South Africa
Pandion haliaetus[b]	UMMZ 225997, T	√	√		Michigan, U.S.A.

[a] Scientific names (Table 5) follow Dickinson (2003) with changes suggested by David and Gosselin (2002). Riesing et al.'s (2003b) proposed generic changes follow a slash after the traditional name.

[b]Sequence data from Lerner and Mindell (2005)

[c]Tissue type: Blood (B), muscle or organ (T), museum toepad (M), feather (F), DNA extract (D), Photo voucher (P)

[d]Genbank sequence used in the mt dataset: NC 003128

[e]Genbank sequences used in the ND6 dataset: NC 003128, AY213011, AY213034, AY213045, AY216914, AY216916-AY216919, AY216921-AY216924, 15990570, 29569538, 29569560; odd numbers 7407009-7407013, 7407023-7407029, 7407057-7407059, 76009021-76009069; even numbers 29569512-29569514, 29569518-29569524, 29569530-29569534, 29569542-29569554, 29569564-29569568, 29569572-29569576

[f]Australian Museum Evolutionary Biology Unit, Sydney (AMS); American Museum of Natural History, New York (AMNH); Academy of Natural Sciences, Philadelphia (ANSP); Natural History Museum, London (BMNH); Field Museum of Natural History, Chicago (FMNH); El Huayco, Lima (HUA); Johannesburg Zoo, Johannesburg (JBZ); Kansas University Natural History Museum, Lawrence (KUNHM); Louisiana State University Natural History Museum, Baton Rouge (LSUMZ); Museum of Southwestern Biology, Albuquerque (MSB); Predatory Bird Centre, Pietermaritzburg (PBC); National Zoological Gardens of South Africa, Pretoria, (PNZ); South Australia Museum, Adelaide (SAM); Tel Aviv University Research Zoo, Tel Aviv (TAU); The Peregrine Fund, Boise (TPF); University of Michigan Museum of Zoology, Ann Arbor (UMMZ); University of Minnesota Raptor Center, Saint Paul

(UMRC); National Museum of Natural History, Washington D.C. (USNM); World of Birds Wildlife Sanctuary, Houtbay (WOB); Zoologisk Museum, Københavns Universitet, Copenhagen (ZMUC).

Melierax, and *Accipiter* and one sample each for two monotypic genera (*Micronisus*, *Urotriorchis*) based on findings of a close relationship between these taxa and *Kaupifalco* using published ND2 and cyt-*b* sequences (Lerner, Mindell, 2005). In order to incorporate more *Buteo* species in our analyses and compare our results to two recent molecular studies, we also sequenced ND6 from 42 of our buteonine samples and analyzed them in a dataset with previously published ND6 sequences from an additional eight *Buteo* species, eight *Buteo* subspecies and three non-*Buteo* buteonine subspecies (Table 1, do Amaral *et al.*, 2006; Riesing *et al.*, 2003b). Samples were identified to the subspecies level based on specimen labels or collection locality and are reported as such in Table 5 and Figure 4. Common names follow the 7th edition of the AOU Check-list of North American Birds and its supplements (AOU, 1988) or The Handbook of Birds of the World (Thiollay, 1994).

Total genomic DNA was extracted from blood or other tissue of contemporary specimens or from toe-pad tissue of museum specimens using a DNeasy Tissue Extraction Kit (QIAGEN Inc.). Lab work involving DNA extraction and PCR set-up from museum samples was conducted in a facility reserved for ancient DNA at the University of Michigan Museum of Zoology using protocols developed for ancient DNAs including multiple extraction and PCR controls (Cooper, Poinar, 2000). PCR amplifications were conducted using primers we designed for Buteoninae as well as published primer sequences for avian mitochondrial cytochrome-*b* (cyt-*b*), ND2, ND6 and the non-repetitive part of the pseudo-control region, and nuclear BF-I7 (primer sequences are reported in Table 6). These genomic regions were chosen for their ability to resolve both recent and deep divergences and their comparability with published

Table 6. Primer Sequences.

Region[a]	Primer ID	Sequence (5-3')
cyt-*b* [b]	H15370.leuc	GAT GTA GGG GAT RGC TGA GA
	L15287.leuc	CYC TYA TAG CAA CYG CCT TC
	H15599.leuc	AGG GAR AAG TAR GGR TGR AA
	L15508.leuc	CAC CTY ACC TTC CTC CAC GA
	L15718.leuc	CCC CAC ACA TCA AAC CAG A
	H15778.leuc	GGG ATT GAG CGT AGR ATR GC
ND2 [b]	H5469.leuc	KAG RAG YGT RGA GGC TGT TG
	L5432.leuc	GCC ATC GAA GCY ACR ATC AA
	H6022.leuc	TGT RGY TRT TTC TTG YTT GG
	L5993.leuc	CAG GCT TCC TRC CCA AAT GR
BF-I7[c]	1H.bf.leuc	TAC TTG GTT GTG GAG CAG CA
	2L.bf.leuc	AGC CAA ATG TCC ATG CAG TT
	2H.bf.leuc	AAC TGA GCA CCT GTC TTC TGA G
	3L.bf.leuc	CAG TAA CAC ATA ATG GGT CCT GA
	3H.bf.leuc	TGG AAG GTG AAG CAG CTA AGA
	4L.bf.leuc	GCA ATT ATC ATT ATG AAC TGC AAG
	4H.bf.leuc	CCA TCC ACC ACC ATC TTC TT

[a] ND6: tPROfwd, tGLUfwd, tGLUrev, YCR2rev (Riesing *et al.*, 2003b)

[b] cyt-*b*, nd2: L14996, H15646, L15560, H16064, L5219, H5766, H6313 (Sorenson *et al.*, 1999)[c] BF-I7: FIB-BI7U, FIB-BIL2, FIB-BIU2, FIB-BI7L (Prychitko, Moore, 2000)

sequences (do Amaral *et al.*, 2006; Lerner, Mindell, 2005; Prychitko, Moore, 2000; Riesing *et al.*, 2003b; Sorenson *et al.*, 1999). PCR products were gel purified using a QIAquick Gel Extraction Kit (Qiagen), directly sequenced from both strands with ABI big dye terminator chemistry and resolved on an ABI 3730 automated sequencer. Sequences were viewed as chromatographs in Sequencher version 4.5 (Gene Codes) and aligned by eye in BioEdit Sequence Alignment Editor (Hall, 1999b).

Corrected sequence divergence (csd) estimates among taxa were calculated using Tamura-Nei (1993) distances in MEGA v2.1 (Kumar *et al.*, 2001). Empirical base frequencies and nucleotide composition bias were calculated in PAUP* (Swofford, 2004). Substitution saturation plots were constructed by codon position and gene for mitochondrial loci in DAMBE using Tamura-Nei genetic distance (1993) and pairwise numbers of transitions and transversions (Xia, 2000).

Phylogenetic reconstruction was carried out using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) separately on each gene or intron and then on multi-locus datasets (see below). MP trees were constructed in PAUP* v.4.0b10 (Swofford, 2004) using heuristic searches with starting trees obtained by random addition of taxa with 10 replicate searches and TBR branch swapping for 1000 bootstrap replicates. Gaps were treated as a fifth state and missing data were treated as uncertainties.

Nonparametric bootstrap ML analyses were conducted on unpartitioned datasets in GARLI v0.94 (Zwickl, 2006). GARLI applies a genetic algorithmic approach similar to GAML (Lewis, 1998). Sequence evolution models are implemented in a manner analogous to that conducted in PAUP* (Swofford, 2004) such that resulting log

likelihood scores are directly comparable to those that would be recovered in PAUP* analyses of sufficient length. We used ModelTest v. 3.7 (Posada, Crandall, 1998) to determine the best fit model for each gene, intron and codon position with the hierarchical likelihood ratio test, all characters equally weighted and a Neighbor-joining starting tree as implemented in PAUP* (Swofford, 2004). The simplest model with the lowest Akaike information criterion (AIC) was chosen for analyses. Bootstrap runs for ML analyses consisted of 500 pseudoreplicate heuristic searches with a GTR + I + G model.

Models with similarly low AIC values were applied separately for each gene, codon position and intron in MrBayes v.3.1.2 (Huelsenbeck, Ronquist, 2001) using four Markov chains sampling every 500 generations for six million generations. For each run the distributions of parameter sampling were visualized and burn-in periods assessed in Tracer v.1.1 (Rambaut, Drummond, 2003). Conservative burn-in periods of 10% were sufficient for all runs. In all cases resulting topologies were identical regardless of the model used and therefore, the simplest model producing the most even distribution of sampling with the greatest number of independent samples (ESS values in Tracer) was chosen for Bayesian inference (Alfaro, Huelsenbeck, 2006).

We assessed four partitioning schemes for joint analyses of cyt-*b* and ND2: one partition including both genes, one partition for each gene (two partitions), one for each codon position (three partitions) and one for each codon position in each gene (six partitions). Similarly, ND6 was assessed as a single partition versus three partitions each corresponding to a different codon position. Joint analyses with the nuclear intron forming a separate partition from the cyt-*b* and ND2 data were performed after the best

partitioning strategy was determined (see below). Parameters were allowed to vary independently for each partition during MrBayes runs. Harmonic mean log likelihoods for each partitioning scheme were calculated using the "sump" command in MrBayes (Table 7). Bayes factors were calculated for each pair-wise combination of partitioning schemes as an objective criterion for determining the best partitioning strategy for final analyses (Brandley *et al.*, 2005). Three independent BI analyses using the partitioning strategy with the highest likelihood score were conducted to test for convergence on similar likelihood scores and topologies.

Results and Discussion

Sequence characteristics and phylogeny.—Numbers of parsimony informative sites and variable but uninformative sites were 487 and 48 out of a total 1120 aligned bps of cyt-*b*, 564 and 74 out of 1047 bps of ND2, 122 and 189 out of 981 bps of BF-I7, and 200 and 42 out of 519 bps of ND6. Empirical base frequencies correspond to those found in other avian studies (mitochondria: A, ~30%; C, ~35%, G, ~10%; T ~24%; BF-I7: A = 31%; C = 17%, G =18%; T = 33%). The χ^2 test of homogeneity showed no significant nucleotide composition bias across study taxa.

Substitution saturation plots (not shown) show nearly linear increases of both transitions and transversions, with a steeper slope for transitions than transversions, except for third base codon positions in ND6 which show some saturation beginning at a genetic distance of ~7%.

An insertion of three adenines was found in *Accipiter nisus* and *A. rufiventris* directly preceding the stop codon of cyt-*b*. Autapomorphic indels in BF-I7 ranged from 1 to 11 base pairs (bps) in length and were found in 12 species. Parsimony informative indels were found for *Circus aeruginosus* and *C. ranivorus* (nine bps deletion);

Table 7. Harmonic mean log likelihood scores for each partitioning scheme.

Partition strategy	**# of partitions**	**Harmonic mean log likelihood**
a. mt dataset		
No partitioning: (cyt-*b* + ND2)	1	-30987.72
Gene: (cyt-*b*), (ND2)	2	-30924.55
Codon position: (cyt-*b* & ND2 codon 1), (cyt-*b* & ND2 codon 2), (cyt-*b* & ND2 codon 3)	3	-29934.30, -29946.06, -29937.33
Gene and codon position: (cyt-*b* codon 1), (cyt-*b* codon 2), (cyt-*b* codon 3), (ND2 codon 1), (ND2 codon 2), (ND2 codon 3)	6	-30049.61
b. ND6 dataset		
No partitioning: (ND6)	1	-4936.33
Codon position: (ND6 codon 1), (ND6 codon 2), (ND6 codon 3)	3	-4767.79, -4767.92, -4768.40

C. aeruginosus, *C. ranivorus*, *A. bicolor*, *A. cirrocephalus*, *A. cooperii* and *A. rufiventris* (one bp deletion), and an insertion for five of these species (one bp not shared by *A. cirrocephalus*). *Leptodon cayanensis, Rostrhamus sociabilis*, *Geranospiza caerulescens*, *Leucopternis schistaceus*, *Harpyhaliaetus solitarius*, and all four *Buteogallus* species share a two bp deletion of TG or GT; and, all four *Buteogallus* species, *H. solitarius* and *L. schistaceus* share a two bp deletion. Due to ambiguity in the DNA sequence it could not be determined if the two bp deletion (TG or GT) described above was synapomorphic for all nine sampled individuals so the two bases were excluded from the analyses for all species. Missing data comprised <10 bps for all individuals except for in cyt-*b* for three individuals: *Butastur indicus* (216 missing bases), *Leucopternis p. princeps* (308 missing bases) and *Buteogallus aequinoctialis* (569 missing bases). No significant difference in topology or likelihood was found between analyses of the mt dataset with and without these sequences; thus, mt analyses shown here include them.

Separate phylogenetic analyses of BF-I7 (not shown) produced a less resolved tree than the other analyses. The relationships among major accipitrid clades were recovered with high support values (Bayesian posterior probability [bpp]=1.00-0.97) as were most sister relationships; however, the branching pattern within the Buteoninae was not resolved beyond finding three separate clades for the buteonine kites and *Geranospiza*, the *Buteogallus* species sister to *Leucopternis schistaceus* and all other Buteoninae (bpp=0.99). Also, the position of *Buteo/Rupornis magnirostris* was unresolved. Separate analyses of cyt-*b* and ND2 also produced trees with several Buteoninae polytomies: (1) a polytomy of three clades: *Busarellus*, *Geranospiza*, *Leucopternis princeps*, *L. plumbeus*, *B./Percnohierax leucorrhous* and *Parabuteo*

unicinctus; the kites; and the *Buteogallus* species, *Harpyhaliaetus* species and *L. schistaceus* and *L. lacernulatus*; and (2) a polytomy of the three remaining buteonine clades (clades diverging after node A in Fig. 3 described below). There were two main differences between the separate ND2 and cyt-*b* analyses: (1) *B./R. magnirostris* diverged before all of the other Buteoninae in the cyt-*b* analyses (bpp=1.00) but in the ND2 analyses was part of an unresolved polytomy with *B./P. leucorrhous*, *Parabuteo unicinctus* and a clade containing the later-diverging Buteoninae (species diverging after node A in Fig. 3 described below, bpp=0.98); and (2) in cyt-*b* analyses the *Butastur* species were part of a five-way polytomy with *Kaupifalco*, a clade of goshawks (genera *Melierax*, *Micronisus* and *Urotriorchis*), a clade of accipiters and harriers (genus *Circus*), and a clade of sea eagles and buteonines (bpp=1.00), while in the ND2 analyses the *Butastur* species were sister to the *Ictinia* species (bpp=0.74). Since single-locus analyses produced overall very similar topologies, we performed joint analyses of cyt-*b* and ND2 and cyt-*b*, ND2 and BF-I7.

Three datasets were assembled. The "mt" dataset included 2066 aligned (i.e. including indels) base pairs (bps) of mitochondrial DNA (1020 bps cyt-*b* and 1046 bps nd2) from 105 Accipitridae individuals representing 76 named species. The "mt + bf" dataset included 3048 bases of aligned combined mitochondrial and nuclear data (the mt dataset appended to 981 bases of BF-I7) for 73 accipitrid taxa representing 56 nominal species. The "ND6" dataset included 519 aligned bases of ND6 for 110 taxa representing 47 nominal species.

For the mt dataset the first codon position was modeled by HKY + I + G and the second and third codon positions were modeled by GTR + I + G. The mt + bf dataset had

four independent partitions: the three mitochondrial partitions described above and a separate partition for BF-I7 using the GTR + G model. For the ND6 dataset the first and third positions were modeled with GTR + G; the second codon position was modeled with HKY + G.

Bayesian consensus trees are shown in Figure 3a for the mt dataset, Figure 3b for the mt + bf dataset and Figure 4 for the ND6 dataset. Posterior probabilities (averages from three independent Bayesian analyses) and MP and ML bootstrap values are shown on the figures. The three different types of analyses produced largely congruent topologies, with the few differences involving nodes resolved with low support in the BI analyses and not resolved in the MP or ML analyses. For instance, the branching pattern of the buteonine kites and *Geranospiza* were unresolved in MP and ML runs and supported by low posterior probabilities in Bayesian analyses (bpp=0.51-0.77).

Nodes were supported with bpp $\geq$ 0.90 for 85% of mt and mt + bf nodes. Analyses resolved nearly all nodes in the mt + bf analyses with higher bpp values than with the mt dataset alone, potentially a result of the larger number of base pairs in the mt + bf dataset. For example, the placement of *Buteo/Rupornis magnirostris* and *B. lineatus* were unresolved in the Bayesian analyses of the mt dataset but were resolved in the mt + bf analyses with high support (bpp=0.96 and 0.98 respectively).

The phylogeny recovered in analyses of the ND6 dataset (Fig. 4) largely agrees with the topologies in Figure 3 except for a polytomy of deeper divergences within and directly preceding the Buteoninae (i.e. placement of *Haliaeetus*, *Busarellus*, *Geranospiza*

Figure 3. Phylogeny for Accipitridae taxa inferred from mitochondrial cyt-b and ND2 (a: mt dataset) and nuclear BF-I7 (b: mt + bf dataset). Topology shown is the Bayesian inference majority rule consensus tree from three independent runs. Bayesian posterior probability (bpp) values (0.50-0.99) are shown above branches; values of 1.00 are denoted by a bolded line leading to the node. Maximum likelihood (ML) values are above nodes, following bpp and/or preceding maximum parsimony (MP) bootstrap values. MP bootstrap values (>50%) are shown in gray-colored italics below branches or following bpp or ML values. Bootstrap values of 100 are denoted by a circle (º) for ML and an asterisk (*) for MP. Dashed lines are extensions of branch lengths; double hashes indicate branches reduced in length. *L. a. williaminae** denotes the type specimen.

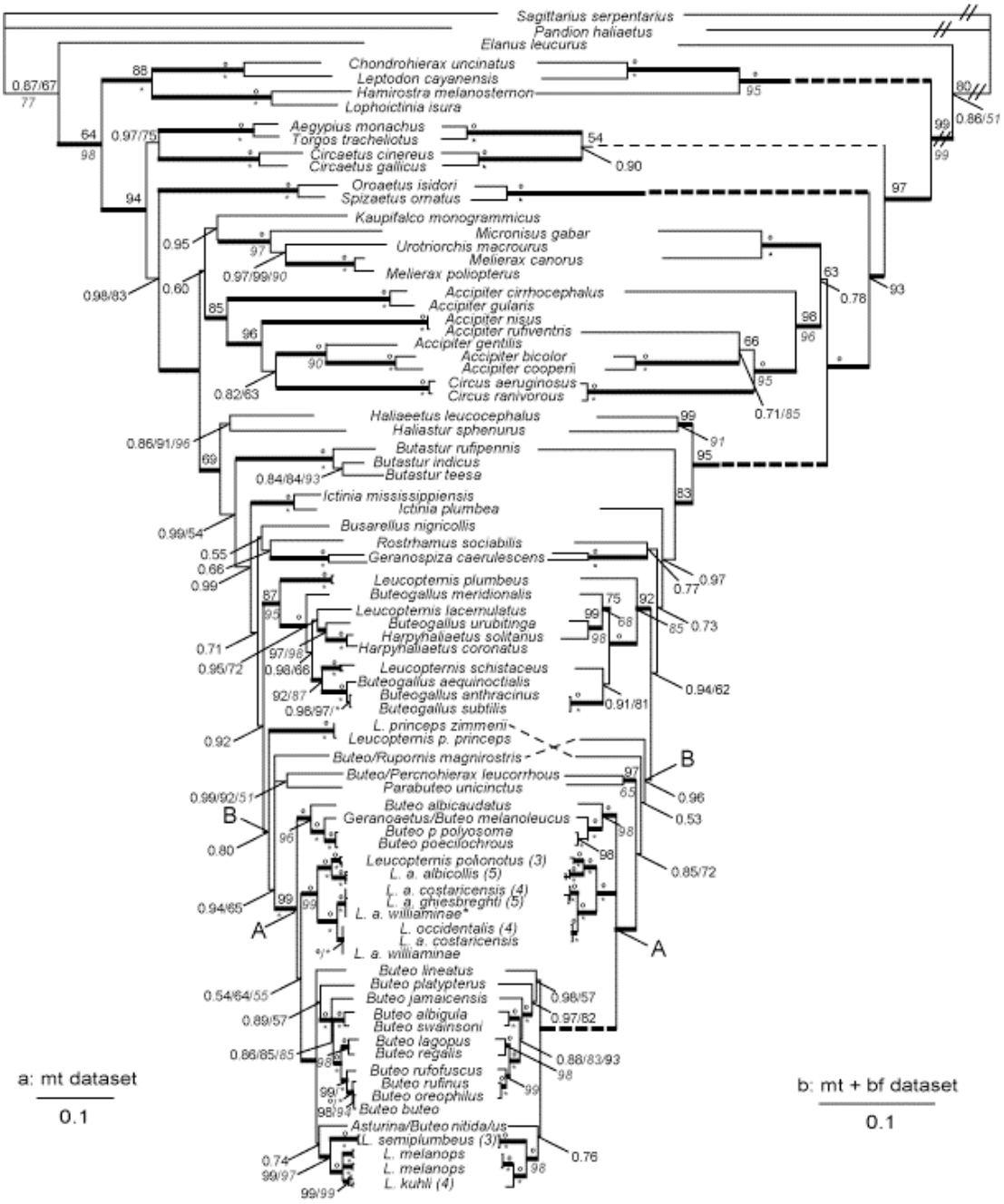


Figure 4. Phylogeny for Accipitrid taxa inferred from ND6 sequences. Topology shown is the Bayesian inference majority rule consensus tree from three independent runs. Bayesian posterior probability (bpp) values (0.50-0.99) are shown above branches and values of 1.00 are denoted by a bolded line leading to the node. Maximum likelihood (ML) values are above nodes, following bpp and/or preceding maximum parsimony (MP) bootstrap values. MP bootstrap values (>50%) are shown in gray-colored italics below the branches or following bpp or ML values. Bootstrap values of 100 are denoted by a circle (º) for ML and an asterisk (*) for MP analyses.

and *Butastur*) which likely results from increased substitution saturation for this gene among older divergences. Within the Buteoninae the positions of *Leucopternis princeps*, *L. plumbeus*, *Buteo p. platypterus* and *Asturina nitida/B. nitidus* were unresolved. The ND6 analyses differ from the mt analyses in that they recover a sister relationship between *L. lacernulatus* and *Buteogallus meridionalis* and show an earlier but unresolved divergence of *B. platypterus.* This could reflect differences in taxon sampling between the analyses, differences between samples of *L. lacernulatus* (ND6 sequence from do Amaral et al. 2006) or differences in their molecular evolution, given that ND6 is the only mitochondrial protein-coding gene encoded by the light strand. Our ND6 analyses were concordant with previous studies (do Amaral *et al.*, 2006; Riesing *et al.*, 2003b) except that we found a sister relationship between *B. r. rufinus* (not B. auguralis as in fig 2 of Riesing *et al.*, 2003b, MP bootstrap=83, Neighbor-joining support=82) and a clade containing *B. brachypterus* and *B. j. japonicus* (bpp=0.90, Fig. 4). Other differences between our analyses and those of Riesing et al. (2003b) involve nodes supported by bootstrap values <50% in their figures.

*Old World Taxa (*Kaupifalco *and* Butastur*) and* Accipiter.—Three species of *Butastur* form a monophyletic group (bpp=1.00, Figs. 3a and 3b) diverging after the sea eagles but before the other sub-buteos, in a clade that is not closely related to *Kaupifalco*. By including representatives from each previously identified clade or subfamily of Accipitridae and expanding sampling of harriers, accipiters and goshawks, we found that *Kaupifalco* is sister to a clade including *Melierax*, *Micronisus* and *Urotriorchis* (bpp=0.95, Fig. 3a) and sister to an *Accipiter* when the goshawks and other non-Buteonine genera were not included (bpp=0.64, Fig. 4). *Kaupifalco* and *Butastur*, both

described as sub-buteos by Amadon in 1982, were later removed from the group by Amadon and Bull (1988). *Kaupifalco* was removed based on observations by Kemp that the "voice and habits" of *Kaupifalco* are more similar to *Melierax* than to sub-buteos. Amadon and Bull also removed *Butastur* from Buteoninae at the same time emphasizing its similarity to *Kaupifalco*. Our results confirm that *Kaupifalco* is indeed more closely related to *Melierax* than to sub-buteos but show that *Butastur* is more closely related to the sub-buteos than to the clade containing *Kaupifalco* and *Melierax*. Therefore, of the two Old World genera, we find support only for *Butastur* as a buteonine genus.

With this expanded sampling, we also found non-monophyly of the genus *Accipiter* when *Circus* species are included. In the mt dataset two *Circus* species are nested within a clade of seven *Accipiter* species (bpp=0.82, Fig. 3a) or three accipiters (bpp=1.00, Fig. 3b). This finding of *Circus* nested within the larger *Accipiter* clade, has not been previously published as far as we know. Earlier studies including both genera, based on smaller sets of taxa and characters with less detailed searches, did not find *Accipiter* polyphyly, but indicated their reciprocal monophyly and a close but non-sister relationship instead (Wink, Sauer-Gurth, 2004; Wink, Seibold, 1996). Our finding of *Accipiter* polyphyly is also supported in analyses with greater sampling of species in both genera that are part of a larger consideration of Accipitridae (*in preparation*).

*Black-collared hawk (*Busarellus nigricollis*).— Busarellus* diverges early within the Buteoninae, after a clade of *Butastur* species and sister to *Geranospiza* and *Rostrhamus* with low support in the mt analyses (bpp=0.55, Fig. 3a) or unresolved with respect to *Butastur*, *Geranospiza* and *Haliaeetus* (bpp=0.95, Fig. 4). Previously proposed sister groups for *Busarellus* include milvine kites and sea eagles (Olson, 1982; Ridgway, 1876),

sub-buteos *Buteogallus* and *Parabuteo* (Brown, Amadon, 1968) or *Hieraaetus* and *Polemaetus* (Holdaway, 1994). We did not find a well-supported close sister relationship for *Busarellus* here, but did confirm its position within Buteoninae.

Relationships among and within New World Buteoninae genera.— Divergence of *Ictinia* follows that of the sea eagles and the genus *Butastur* (bpp=0.99, 0.97, Fig. 3). *Rostrhamus* is sister to *Geranospiza* but with low support (bpp=0.66, 0.77, Fig. 3). With nearly complete sampling in *Buteogallus* and *Leucopternis*, we confirmed their non-monophyly (do Amaral *et al.*, 2006; Lerner, Mindell, 2005). Both *L. schistaceus* and *L. plumbeus* had been placed in the genus *Urubitinga* (Ridgway, 1876; Sharpe, 1874), now synonymous with *Buteogallus* (AOU, 1988; Peters, 1931), based on morphological similarities with *B. anthracinus* and *B. urubitinga*. Here we find that these two *Leucopternis* species are indeed more closely related to *Buteogallus* species than to other *Leucopternis* species, however they are not sister taxa as proposed (Amadon, 1982a).

The clade including some *Leucopternis*, all *Buteogallus* and both *Harpyhaliaetus* species shows a well-supported split between species that are dependent on aquatic habitats such as mangroves, marshes, forest and wetlands (*B. aequinoctialis*, *B. anthracinus*, *B. subtilis* and *L. schistaceus*) and mostly forest or open-vegetation habitats (L. lacernulatus, B. urubitinga, H. solitarius and H. coronatus, Fig. 2, bpp=0.98, 1.00, Ferguson-Lees, Christie, 2001).

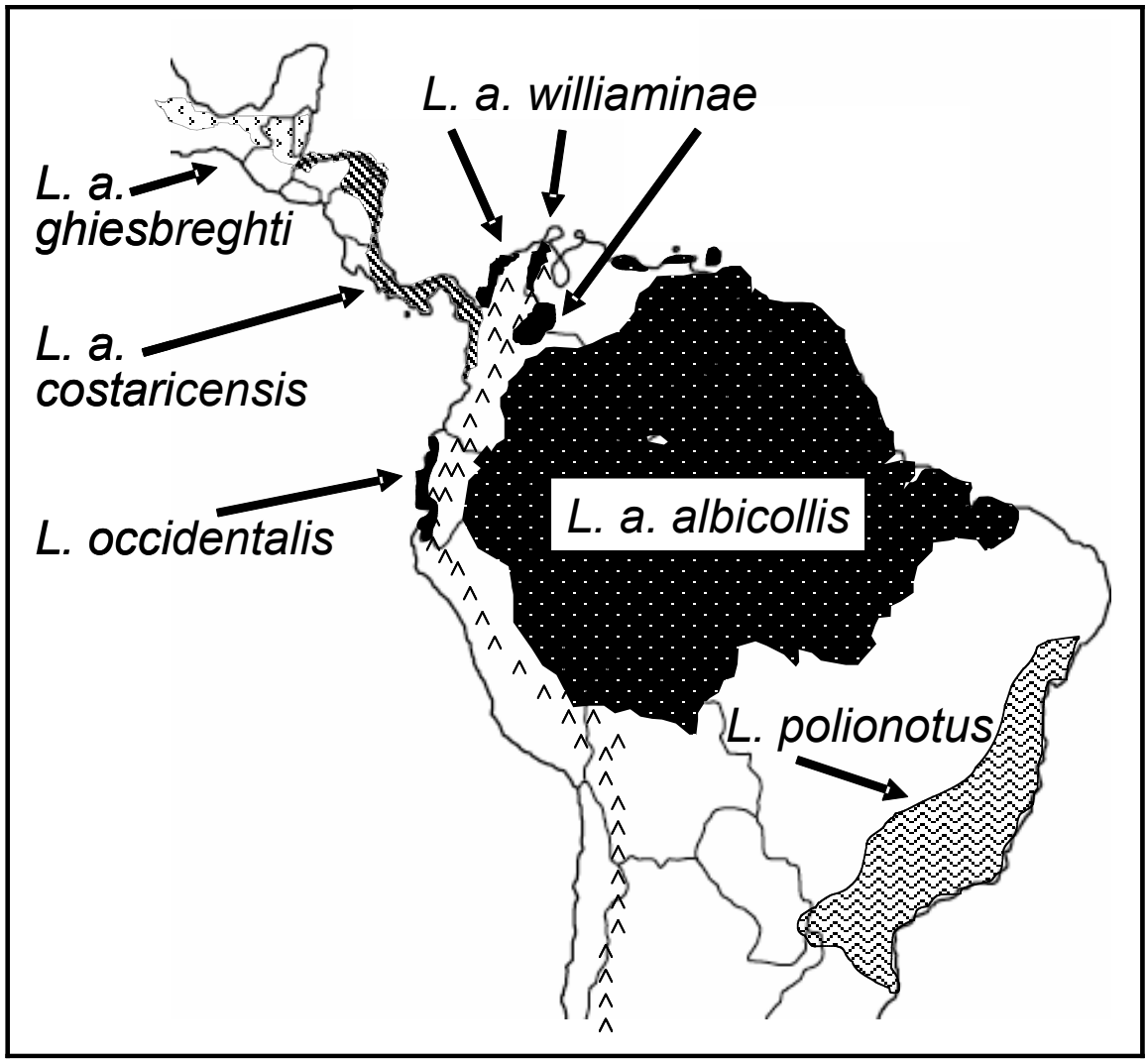
Leucopternis species are members of four different non-sister clades within the Buteoninae (Fig. 3, two species unresolved in Fig. 4). We found that *L. princeps* is more closely related to a large clade of *Buteo* and other *Leucopternis* taxa (bpp=0.80, 0.53 Fig. 3; unresolved in Fig. 4) than to a clade of *Buteogallus*, *Harpyhaliaetus* and *Leucopternis*

(figure 1 bpp=0.68, bootstrap=58, do Amaral *et al.*, 2006). The lack of resolution for *L. princeps* in Fig. 4 and the difference between Fig. 3 and the results of do Amaral et al. (2006) likely reflect differences in the size and informativeness the datasets.

Genetic divergence among Buteogallus subtilis, B. anthracinus *and* B. aequinoctialis.—The Mangrove Black Hawk (*B. subtilis*) and Common Black Hawk (*B. anthracinus*) individuals we sampled had identical BF-I7 sequence, only one base pair difference in cyt-*b* and another single difference in ND2, while the Rufous Crab-hawk (*B. aequinoctialis*) was different from both of these species at 20 mitochondrial bases (2% csd). *B. subtilis* has been considered a subspecies of *B. anthracinus* and a member of a superspecies with *B. aequinoctialis* (Brown, Amadon, 1968). Given that these three taxa are distributed in adjacent and sometimes overlapping ranges in similar habitat on the Atlantic and Pacific coasts and islands of the New World tropics, potential for interbreeding exists and broader geographic sampling is needed before taxonomic revisions can be made.

*Non-monophyly of nominal White Hawk subspecies (*Leucopternis albicollis*).*—We sampled two to five (average=4) individuals from the broad geographic range of each White Hawk subspecies, the Grey-backed Hawk (*L. occidentalis*) and Mantled Hawk (*L. polionotus*; Figure 5). The White Hawk was not monophyletic, with the nominate form (*L. a. albicollis*) more closely related to *L. polionotus* than to other *L. albicollis* subspecies (bpp=1.00, Fig. 3; bpp=0.60, Fig. 4). *L. a. albicollis* individuals are 2.3% (mt csd) divergent from *L. polionotus* individuals, a value similar to that found for other Accipitrid sister taxa (0.5-3.8% csd among Gyps species, Johnson *et al.*, 2006; 95-98% sequence similarity for booted eagles, Lerner, Mindell, 2005).

Figure 5. Geographical distribution of White Hawk (Leucopternis albicollis) and related taxa. Compiled from published descriptions and maps (Ferguson-Lees, Christie, 2001; Hilty, 2003; Hilty, Brown, 1986; Howell, Webb, 1995; BirdLife International, 2004; Jones, 2003; Land, 1970; Monroe, 1968; Sick, 1993; Slud, 1964; Thurber *et al.*, 1987; Wetmore, 1965).



The three trans-Andean (i.e. west of the Andean cordillera) subspecies of L. albicollis and L. occidentalis share mt haplotypes (Fig. 3a) and exhibit gradation of plumage coloration from nearly all white birds in the north (L. a. ghiesbreghti) to heavy black coloration on the heads and wings of southern birds (L. occidentalis; Lerner, Klaver and Mindell, unpublished). Individuals from the most northern subspecies, L. a. ghiesbreghti, formed a clade sister to representatives of L. occidentalis, the most southern species; however, individuals from two White Hawk subspecies occurring in the center of the trans-Andean range for these taxa (L. a. costaricensis and L. a. williaminae from southern Central America and northern South America) were found in both clades. The subspecies L. a. williaminae has a very small range and is known from only a few museum specimens (the type specimen is denoted by "*" after the name on Fig. 3). The two clades identified in trans-Andean birds do not strictly correspond to current taxonomy, geography or plumage coloration. These clades diverge by an average 1.2% (mt csd), which is similar to but on the low end of that observed between other Accipitridae sister species pairs (Johnson *et al.*, 2006; Lerner, Mindell, 2005). Members of the trans-Andean clades differ from their sister clade containing L. a. albicollis and L. polionotus by 4.4% (average mt csd).

Analyses with greater sampling of individuals are needed, however, the current set of relationships based on mitochondrial data (Figs. 3a and 4 but not Fig. 3b) support *L. a. albicollis* being recognized as *L. albicollis* and *L. a. costaricensis, L. a. ghiesbreghti,* and *L. a. williaminae* as one or more distinct species. Four to six endangered Grey-backed Hawks (*L. occidentalis*) form a monophyletic (Fig. 4) or unresolved group nested within a clade of *L. a. costaricensis, L. a. ghiesbreghti,* and *L.*

williaminae individuals (Fig. 3). None of these clades were recovered with nuclear intron data alone. This may reflect differences in expected coalescence times among maternally versus biparentally inherited loci, especially if these divergences are recent and/or the effective population sizes are large (Hudson, 1990). Using more variable loci, additional specimens and population genetic methods could help in further taxonomic assessment and to distinguish between alternative hypotheses such as incipient speciation, secondary contact or isolation by distance for this clade. Given the status of the small and isolated populations of *L. occidentalis*, such analyses could be useful for conservation programs..

Genetic divergence between Leucopternis kuhli *and* L. melanops.— White-browed Hawks (*L. kuhli*) and Black-faced Hawks (*L. melanops*) are similar in appearance and are considered separate but closely related species (Amadon, 1982b; Hellmayr, Conover, 1949). There were no shared mt or BF-I7 haplotypes between the species and with mt data they are 1.8% divergent from each other. The polytomy in Figure 3a, however, precludes strong conclusions in this regard. The four *L. melanops* individuals are nearly as divergent from each other as they are from *L. kuhli* individuals, with 1.4% average csd, while the average csd among four conspecific *L. kuhli* individuals is 0.56%. Using the more variable ND6 dataset plus additional pseudo control region sequence, two *L. melanops* individuals from Peru are 0.24% divergent from each other, and on average 2.04% divergent from a Peruvian *L. kuhli*. These values are similar to but on the low end of those found between other closely related Accipitridae species (see above).

Although originally described as allopatric, potential for hybridization exists as individuals of *L. melanops* have been trapped simultaneously with *L. kuhli* south of the

Amazon river (Olalla collections of 1930 at the American Museum of Natural History [AMNH], and recent trappings described in Barlow *et al.*, 2002). The two species, however, appear identifiable by plumage: about 20 specimens of each species examined at the AMNH were distinct in plumage with no intermediate plumage types observed. Given the high level of genetic diversity within *L. melanops*, the lack of resolution of the mitochondrial dataset and potential for hybridization, further analysis of these two species or "superspecies" is warranted.

Phylogeny and taxonomy of the genus Buteo.— In Figure 3 all members of the nominal genus *Buteo* diverge after the node labeled "B." Following the early divergence of *L. princeps* and *B./R. magnirostris*, a sister relationship between *B./Percnohierax leucorrhous* and *Parabuteo unicinctus* is supported (bpp=0.99, 1.00, Fig. 3; bpp=0.82, Fig. 4). The remaining *Buteo* species fall into two clades: (1) *B. albicaudatus*, *Geranoaetus melanoleucus*, *B. poecilochrous* and *B. polyosoma* and (2) all others (11 species in Fig. 3, 18 species in Fig. 4). The positions of *B. lineatus*, *Asturina nitida*/*Buteo nitidus* and *B. jamaicensis* have not been resolved or well-supported previously (nodes III [MP bootstrap=58, Neighbor-joining support=90] and IV [support values <50] in Riesing *et al.*, 2003b). In Figure 3 we find that the divergence of *B. lineatus* (bpp=0.98, Fig. 3b) is followed by that of *B. platypterus* (bpp=0.97, Fig. 3b), and *Asturina nitida/B. nitidu*s is more closely related to several species of *Leucopternis* than to these two *Buteo* species (bpp=0.74, 0.76, Fig. 4; node III in Riesing et al. 2003). We also find that divergence of *B. jamaicensis* (bpp=1.00, Fig. 3) is followed by divergence of the sister species *B. albigula* and *B. swainsonii* (bpp=1.00, 0.88, Fig. 3; bpp=0.65, Fig. 4). Within the Buteoninae we find that earlier divergences correspond to taxa with New World

distributions followed by the sister pair of Nearctic *B. regalis* and circumpolar *B. lagopus* (bpp=1.00, Figs. 3 and 4) and all Old World taxa diverging last (Figs. 3 and 4; see also Riesing et al. 2003).

We support the idea that taxonomy should reflect phylogeny. In that spirit, one proposal for redefinition of the genus *Buteo* includes all species descended from node A (Figs. 2 and 3 and in Riesing *et al.*, 2003b). With the dataset used by Riesing et al. (2003) this proposal would have required changing the generic names for three species (*Asturina nitida* to *Buteo nitidus, B. magnirostris* to *Rupornis magnirostris* and *B. leucorrhous* to *Percnohierax leucorrhous*). Delimiting the genus *Buteo* at node A of Fig. 3 in our analyses would require changing the generic names for an additional six species of *Leucopternis* as well as for *Geranoaetus*. We suggest, however, that delimiting *Buteo* earlier in the tree at node B (Fig. 3) is preferable, comprising a single clade including all current members of the genus *Buteo* sampled in both studies, and involving a change in genus name for two more species (*Parabuteo unicinctus* and *L. princeps*).

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Chapter 4

Genetic divergence among Harpy eagles (*Harpia harpyja*) in Central America and South America

Top predators play a crucial role in maintaining the trophic interactions of terrestrial systems (e.g. Gutiérrez *et al.*, 1997; Letourneau, Dyer, 1998a; Letourneau, Dyer, 1998b; Strong *et al.*, 2000; Van Bael *et al.*, 2003). In particular, vertebrate carnivores have the strongest direct effects on herbivores and strongest indirect effects on limiting plant damage (Schmitz *et al.*, 2000). These "top-down" effects have been studied in a Venezuelan tropical forest where the construction of a hydroelectric dam formed a giant lake, Lago Guri that isolated and fragmented once-continuous tropical forest habitat into a series of islands where vertebrate top-predators were excluded. In the absence of predators such as harpy eagles (*Harpia harpyja*), jaguars and pumas (*Felis concolor*), the Lago Guri islands have experienced population explosions of one or more different herbivores and seed predators including howler monkeys, iguanas, rodents and ants (Lopez *et al.*, 2005; Terborgh *et al.*, 2001). These islands in turn show vast reductions in plant species diversity including an 80% reduction in the recruitment of canopy trees (Terborgh *et al.*, 2006). Similarly, while bird density increased, diversity decreased (Terborgh *et al.*, 1997).

The dramatic alterations of the Lago Guri islands provide a glimpse of the potential future for Neotropical forests as populations of many top predators decline. Harpy eagles are the largest extant birds of prey in the New World feeding on sloths, monkeys and

other arboreal mammals of lowland rainforests (Eason, 1989; Fowler, Cope, 1964; Rettig, 1977; Rettig, 1978). Their current distribution spreads from southern Mexico to east-central Brazil (del Hoyo *et al.*, 1994), however destruction of rain forest habitat, particularly extensive in Central America (FAO, 2006), has fragmented their distribution and has likely contributed to local extinctions throughout their range (del Hoyo *et al.*, 1994; Vargas *et al.*, 2006).

As with other Neotropical top-predators such as jaguars (*Panthera onca*), ocelots (*Leopardus pardalis*) and margays (*L. wiedii*), harpy eagle population reductions are attributed to slow rates of reproduction, dependence on high-quality rainforest habitat and human persecution (Collar *et al.*, 2001; Eizirik *et al.*, 1998; Eizirik *et al.*, 2001). The World Conservation Union lists the harpy eagle as near-threatened (IUCN, 2006) and conservation programs are underway in many Latin American countries. Since genetic diversity is important for the persistence of populations (Frankham, 2005; Reed, Frankham, 2003; Spielman *et al.*, 2004), estimates of genetic variability and demographic parameters for species threatened with extinction are valuable for conservation efforts (O'Brien, 1994).

In this study we use coalescent and phylogenetic based analyses and quantitative test statistics of molecular sequence data to reconstruct the population demographic history of the harpy eagle. In particular, we quantify levels of genetic diversity, assess the possibility of gene flow among geographic populations, and estimate relative effective population sizes using mitochondrial control region sequence data from harpy eagles collected from a broad geographic range across 12 Neotropical countries.

Methods

Samples.—Harpy eagle samples were collected from all of the South American and most Central American countries where they have not been extirpated (Table 8). Because of the larger area of intact rain forest habitat in Panama as compared to other Central American countries and the availability of samples from collaborators associated with The Peregrine Fund, Panamanian samples dominate the Central American dataset. The majority of samples are from contemporary specimens collected after 1960; however, ten specimens were collected between 1902 and 1938 and one sample was collected in 1868. The samples obtained from museum collections were used to represent geographic areas where harpy eagles have been extirpated (e.g., Mexico) or from countries where the current export of tissue samples is difficult (e.g., Brazil)., The Crested Eagle (*Morphnus guianensis*), the sister species to the harpy eagle (see Lerner & Mindell 2005), was included as an outgroup for the phylogenetic analyses.

DNA sequences.—DNA was extracted from blood, feathers, and organ tissues using a DNeasy Extraction Kit (QIAGEN Inc.), with 30 μl of 100 ng/ml dithiothreitol (DTT) added to the extraction buffer when working with feathers. DNA extraction from museum toe pads was performed as described in Lerner and Mindell (2005) and conducted in a facility reserved for ancient DNA work at the University of Michigan Museum of Zoology using protocols developed for ancient DNAs including negative extraction and blank amplification controls (Cooper, 1994; Gilbert *et al.*, 2005).

Table 8. Sample information for harpy eagles (*Harpia harpyja*) and one outgroup (*Morphnus guianensis*) analyzed in this study.

Source name[1]/ Cat. No.	Collection Date	Collection Locality	Tissue Type
Bell-07	1994	Venezuela	Liver
WFVZ 10471	1962	Mexico	Feather
TPF Freedom	1997	Unknown	Blood
TPF 94SD	1997	Colombia	Blood
TPF 095[2]	1997	Ecuador	Blood
TPF GBGrey	1997	Venezuela	Blood
TPF FRBL	1997	Panama	Blood
TPF CHEY	1997	Unknown	Blood
TPF CRAWL	1997	Venezuela	Blood
TPF COCA	1997	Ecuador	Blood
TPF OLIVA	1997	Venezuela	Blood
TPF OLAFA	1997	Ecuador	Blood
TPF MilZoo	1997	Ecuador	Blood
WFVZ 001	1999	Guyana	Feather
WFVZ 002	1999	Guyana	Feather
LSUMZ 111050	1982	Peru	Feather
TPF HE-021	2004	Panama	Blood
TPF 008	2004	Panama	Blood
TPF HE-018	2003	Panama	Blood
UMMZ 239465	2003	Panama	Blood
TPF 020	2004	Panama	Blood
TPF HE-015	2004	Panama	Toepad
TPF HE-016	2003	Panama	Toepad
TPF HE-007	2004	Panama	Feather
TPF HE-01	2004	Panama	Feather
TPF HE-017	2004	Panama	Feather
TPF HE-014	2004	Panama	Feather
TPF HE-010	2004	Panama	Feather
TPF HE-001	2003	Panama	Feather
TPF CRE-005	2002	Panama	Feather
TPF HE-013	2004	Panama	Feather
TPF HE-012	2004	Panama	Feather
TPF HE-006	2003	Panama	Feather
TPF HE-003	2002	Panama	Feather
TPF HE-008	2004	Panama	Feather
TPF HE-004	unknown	Panama	Feather
TPF HE-002	2002	Panama	Feather
TPF HE-009	2003	Panama	Feather
UMMZ 239466	2005	Panama	Tissue
UMMZ BD-8225	2005	Panama	Tissue

UMMZ 239471	2005	Panama	Tissue
UMMZ PAN-01	2003	Ecuador	Feather
SDZ 402158	2004	Unknown[3]	Feather
SB	2003	Guyana	Feather
KUNH 24802	unknown	Mexico	Toe pad
FMNH 260141	1964	Surinam	Toe pad
FMNH 264326	1965	Surinam	Toe pad
FMNH 371026	1977	Ecuador	Toe pad
FMNH 104888	1938	Guyana	Stomach Contents
FMNH 32150	unknown	Guyana	Toe pad
USNM 54224	1868	Mexico	Toe pad
USNM 193559	1902	Nicaragua	Toe pad
USNM 253473	1917	Brazil	Toe pad
JMM-A-3224	1960	Guyana	Toe pad
MCZ 58503		Costa Rica	Toe pad
ROM 94251	1963	Guyana	Toe pad
LSUMZ 31239	1963	Peru	Toe pad
LSUMZ 35120	1964	Peru	Feather
LSUMZ B-51351	1963	Peru	Toe pad
LSUMZ B-51352	1963	Peru	Toe pad
LSUMZ 51268	1946	Bolivia	Toe pad
AMNH 102432	1911	Nicaragua	Toe pad
AMNH 238836	1932	Peru	Toe pad
AMNH 406859	1931	Peru	Toe pad
AMNH 429102	1935	Brazil	Toe pad
TPF CUBL.2IH	1997	Ecuador	Feather
AMNH 272336	1932	Venezuela	Toe pad
HUA, *Morphnus guianensis*	2003	Peru	Blood

[1]Bell Museum of Natural History (BELL), Western Foundation of Vertebrate Zoology (WFVZ), Louisiana State University (LSUMZ), The Peregrine Fund (TPF), Royal Ontario Museum (ROM), Museum of Comparative Zoology (MCZ), American Museum of Natural History (AMNH), Joseph Moore Museum (JMM), El Huayco, Peru (HUA), San Diego Zoo (SDZ), Sue Boinski (SB), Western Foundation of Vertebrate Zoology (WFVZ), University of Michigan Museum of Zoology (UMMZ), National Museum of Natural History (USNM), Field Museum of Natural History (FMNH)

[2]sibling of HH-41, excluded from all analyses

[3]likely origin is Columbia

We sequenced 417 bp of domain I of the mitochondrial control region using either two or four primers (LDL-1, HDL-3, LDL-3 and HDL-1) depending on whether we were working with contemporary or museum samples, respectively (Table 9). PCR amplification was performed using Platinum Taq polymerase (Invitrogen). Amplification products were purified on 1.5% low-melting point agarose gels, excised and recovered with a Gel Extraction Kit (Qiagen). PCR products were used for direct sequencing with ABI PRISM Big Dye Terminator Reaction Kits (Applied Biosystems) and resolved on an ABI 3730 automated sequencer. Sequences were aligned by eye in BioEdit Sequence Alignment Editor (Hall, 1999b).

Analyses.—Samples were grouped by geographic regions (see Table 10) to test possible effects of barriers to gene flow such as mountains and discontinuities of lowland forest habitats. The Andean mountains bisect the rainforest habitat of Panama and western Colombia from the Amazon basin forming a barrier known to limit gene flow in a variety of organisms (e.g. passerine birds, Bates *et al.*, 1998; butterflies, Brower, 1994; howler monkeys, Cortes-Ortiz *et al.*, 2003; rainforest trees, Dick *et al.*, 2003). Harpy eagles, however, have large geographic ranges and are capable of traveling long distances by flight (e.g. harpy eagles released in Brazil have traveled over 300 km from the release site, Curti, 2007). Therefore, few geographic features may actually act as barriers to gene flow for this species; although geographic structure has been identified in several felid species having large home ranges in Neotropical forests (Eizirik *et al.*, 1998; Eizirik *et al.*, 2001). To investigate regional gene flow, we identified two major regions

Table 9. Primer sequences used for the amplification of the mitochondrial control region in harpy eagles.

Primer ID	Sequence (5'-3')
LDL-1	CCCATTATCATGCACTATTCTAGG
HDL-1	GAGCAAGGTCGTAGGACTAACC
HDL-3	ATAACCTGGTCCGACAYACG
LDL-3	CGGATATTCTTGGGGACAAA

(1) Central America (including the Darien of Panama and western Colombia), and (2) South America. Within the Central American region we grouped individuals from Mexico, Nicaragua and Costa Rica separately from Panamanian birds based on the lack of continuity of lowland tropical forest between these areas and evidence of corresponding geographic structure in other organisms (Dick *et al.*, 2003; Eizirik *et al.*, 1998; Eizirik *et al.*, 2001). Within South America we defined a north-eastern subgroup (Guyana, Surinam and Venezuela), a western subgroup (Ecuador, Peru, eastern Colombia) and a southern subgroup (Brazil and Bolivia). These subgroups also correspond to geographic division identified in other Neotropical organisms (see above).

The level of genetic diversity within regions and subgroups (defined above) was estimated by calculating the number of haplotypes, haplotype diversity (h) and nucleotide diversity (π) using the program Arlequin v. 3.0.1 (Excoffier *et al.*, 2005). To visualize the relationships among haplotypes we inferred a median-joining network (Bandelt *et al.*, 1999) with genetic distance parameter $e = 0$, equal weights for transitions and transversions and all character sites, and with *Morphnus guianensis* as an outgroup using the program NETWORK, v. 4.2 (available at www.fluxus-engineering.com). The median-joining approach returns a network that corresponds most closely to the strict consensus

of maximum parsimony trees found in phylogenetic analyses (Cassens *et al.*, 2003).

Relationships between harpy eagle haplotypes in Central and South America were also estimated by maximum likelihood in PAUP*. A heuristic search with 10 random addition sequence replicates and 100 bootstrap replicates under the HKY model of sequence evolution (Hasegawa *et al.*, 1985) selected using ModelTest (Posada, Crandall, 1998) was performed with and without constraining monophyly of Central American and South American individuals. The significance of the difference in resulting likelihood scores was evaluated using a parametric bootstrap where 1000 data matrices of 400 bases were simulated under the HKY model in Mesquite (Maddison, Maddison, 2005). Each simulated dataset was subjected to a maximum likelihood analysis as described above, with and without monophyly constraints. The difference in likelihood scores between these runs comprised the null distribution against which the likelihood value from the harpy eagle dataset was tested.

The degree of population differentiation among regions was estimated with F_{ST} using the infinite allele model. Partitioning of genetic variance among geographic regions, among subgroups within regions and within subgroups was determined with hierarchical analyses of molecular variance using haplotype frequencies (AMOVA, Excoffier *et al.*, 1992), and significance was determined based on 16002 non-parametric permutations. AMOVA and F_{ST} calculations were performed in Arlequin v. 3.0.1 (Excoffier *et al.*, 2005).

Demographic histories of harpy eagles in Central and South America were evaluated with three approaches: standard quantitative test statistics, mismatch distributions and coalescent-based estimations.

To test for genetic signatures of recent population size changes, Fu's test of neutrality (*Fs*, Fu, 1997), Tajima's *D* (Tajima, 1989a) and Fu and Li's *F* and D^F test statistics (Fu, Li, 1993) were compared among Central and South American regions and subgroups. Both Fu's *Fs* and Tajima's *D* use the infinite site-model without recombination to test for departures from selective neutrality and population equilibrium for intraspecific data. Fu's *Fs* uses information from the haplotype distribution and is particularly sensitive to population demographic expansion where low *Fs* values indicate an excess of singleton mutations usually due to expansion (Fu, 1997). Tajima's *D* uses the average number of pairwise differences and number of segregating sites in the intraspecific DNA sequence to test for departure from neutral expectations, generally assuming negative values in populations that have experienced size changes, especially expansions, or for sequences that have undergone selection. In populations that have undergone recent bottlenecks or have genetic substructure, values for Tajima's *D* are typically positive (Tajima, 1989b). Fu and Li's *F* and D^F compare mutations observed within a population to an outgroup sequence, using information from the number of recent mutations as evidence of recent expansion. Negative values of Fu and Li's *F* and D^F indicate an excess of rare alleles and recent mutations that are consistent with an increase in population size or positive selection, whereas positive values reflect an excess of alleles at intermediate frequency that can result from population bottlenecks or balancing selection (Fu, Li, 1993). Fu's *Fs* and Tajima's D were calculated in Arlequin v. 3.0.1with 1000 random permutations and Fu and Li's *F* and D^F were estimated in DNAsp (Rozas *et al.*, 2003).

The demographic history of each region was investigated by comparing the shape

of their respective mismatch distributions calculated in Arlequin v. 3.0.1 to shapes expected in stationary and expanding populations. For samples drawn from populations that are at demographic equilibrium, mismatch distributions are usually multimodal (Slatkin, Hudson, 1991). Populations that have experienced recent expansions, on the other hand, typically produce a unimodal distribution, although a similar shape may be result from a bottleneck, making these two processes difficult to distinguish (Rogers, Harpending, 1992). The distribution of the sum of squared differences (SSD) between the observed mismatch distribution for each region and a mismatch distribution estimated under a model of population expansion is used as a test statistic where a significant SSD value indicates departure from a model of sudden population expansion (Schneider, Excoffier, 1999). To estimate the time of expansion (t) we converted the parameter τ, estimated from the mismatch distribution, using the equation $\tau = 2\mu t$ (Rogers, 1995). Confidence intervals for τ were calculated using a parametric bootstrap approach (Schneider, Excoffier, 1999).

The migration rate between regions and relative effective population sizes ($\theta = N_{ef}\mu$, where N_{ef} is the female effective population size and μ is the mutation rate per locus per year) were estimated with MIGRATE (v. 2.1, Beerli, Felsenstein, 1999; Beerli, Felsenstein, 2001). Estimates of μ generated from default settings were used as initial starting points for final runs. Three final runs were conducted to check for convergence upon similar values using the following parameters: 10 short chains of 100 000 steps and two long chains of 20 000 000 steps with sampling every 100 steps and a burnin of 200 000 steps. Likelihood ratio tests were performed in each final run to evaluate the support for symmetric versus asymmetric migration.

To evaluate the differing scenarios of recurrent gene flow and ancestral polymorphism we used two coalescent-based methods that simultaneously estimate gene flow and divergence times. Estimates of the female effective population sizes (($\theta_T = 2N_{ef}\mu$, where N_{ef} is the female effective population size and μ is the mutation rate per locus per year), migration between the regions ($M=2N_{ef}\mu$), time since divergence ($T= t/2N_{ef}$ where t is the generation time) and time to most recent common ancestor (TMRCA=$t\mu$) were estimated using a Bayesian likelihood approach assuming the HKY finite sites model in the program MDIV (Nielsen, Wakeley, 2001; Nielson, 2002). We conducted three independent runs using different random number seeds to evaluate convergence upon similar values of the modes in posterior distributions. Upper bounds for M, θ_T, and T were set to ten. The posterior distribution of T approached but did not reach zero in the upper portion of the distribution, so additional analyses were performed with an upper bound of 20. The posterior distribution for runs with this larger prior remained level rather than converging upon zero, so runs using the smaller prior are reported here. The length of the markov chain was set to 2.5 million generations with a burnin of 500,000 generations. Posterior distributions for the parameters were plotted and the mode of the posterior distribution was selected as the best estimate with the exception of the parameter T, where the point with the highest likelihood value was used.

To convert parameter estimates generated by MIGRATE and MDIV to biologically informative values, an estimate of the neutral mutation rate per generation is needed for the control region. A mutation rate has not been calibrated for any Accipitridae species, so we used a range of mutation rates calculated for the entire control region in grouse (4.54-12.54% [average 7.23%] divergence per million years, Drovetski, 2003) which is

similar to that found for the most variable part of the control region in diving ducks (8.8%, Sorenson, Fleischer, 1996). When converting maximum likelihood estimates and modes of parameters we used the average mutation rate of 7.23% divergence per million years. To incorporate the effect of uncertainty around the mutation rate, we used the upper and lower estimates of the mutation rate (4.54-12.54%) to calculate wider credibility intervals (CI) than if we had simply used the average mutation rate.

Results

Sequence characteristics.—Control region sequences were generated for 66 harpy eagles and a single representative from the outgroup, *Morphnus guianensis* (Table 8). There were 32 harpy eagles sampled from Central America, 31 from South America and three for which the locality was unknown. Twenty-two harpy eagle haplotypes were identified from a total of 21 variable sites, all of which were transitions (Table 11). There were four and 13 unique haplotypes in Central and South America, respectively, and three haplotypes were shared between regions. These shared haplotypes were observed only in birds from Panama and northern South America (e.g., Colombia, Venezuela, Guyana and Ecuador). Two additional haplotypes were shared by individuals sampled in South America and individuals of unknown origin. The majority of haplotypes for individuals sampled in South America were represented by only one or two individuals, with exception of the three haplotypes shared by Central and South American individuals and one haplotype shared by two South American individuals and a bird of unknown origin.

The South American region possessed higher haplotype diversity (0.9548 ± 0.0184; h ± s.e.) than Central America (0.7681 ± 0.0529). Total nucleotide diversity was similar between the regions (South America, 0.008 ± 0.005; Central America, 0.005 ± 0.003). Of the 22 harpy eagle haplotypes sampled, 18 were found in the South American

region while only seven haplotypes occurred in samples from Central America.

Population subdivision.—Significant genetic subdivision between Central and South America was identified with an F_{ST} value of 0.230 ($p<0.0001$). All pairwise comparisons of subgroups within regions had significant F_{ST} values except Mexico-Costa Rica-Nicaragua versus Panama (Table 10). Analysis of molecular variance (AMOVA) showed substantial variation among regions (10.02%) and among subgroups within regions (22.27%) with the majority of genetic variation observed within subgroups (67.71%).

Table 10. Matrix of pairwise F_{ST} values for geographic subgroups.

Geographic subgroup (n=sample size)	Mexico-Costa Rica-Nicaragua	Panama	Colombia-Ecuador-Peru	Venezuela-Surinam-Guyana
Mexico-Costa Rica-Nicaragua (n=6)				
Panama (n=26)	0.10, n.s.			
Colombia-Ecuador-Peru (n=14)	0.33**	0.11*		
Venezuela-Surinam-Guyana (n=14)	0.61**	0.39**	0.19**	
Brazil-Bolivia (n=6)	0.57**	0.30**	0.29**	0.42**

* $p<0.05$ **$p<0.01$ n.s.= not significant

The relationships among haplotypes in both Central and South America including three samples of unknown origin are shown in a median-joining network (Figure 6). The shortest tree length had 90 nucleotide substitutions. Among samples from Central America, five haplotypes cluster within one to two mutational steps from each other, while the remaining two haplotypes are a minimum of four and six nucleotide substitution steps from this cluster. All seven haplotypes recovered in Central American samples were found in at least one Panamanian bird.

Figure 6. A median-joining network depicting the relationships between South American (solid nodes) and Central American (hatched nodes) harpy eagle haplotypes. Haplotypes from three samples of unknown origin are represented by white sections within nodes. Node sizes are proportional to haplotype frequencies. There were 62 mutational steps between the nearest node (an inferred intermediate haplotype) and the outgroup, *Morphnus guianensis* (gray diagonally lined node). Four small gray circles represent inferred intermediate haplotypes not represented by any sampled eagles. Lines connecting ingroup nodes represent a single nucleotide change with hash marks denoting additional single nucleotide changes.

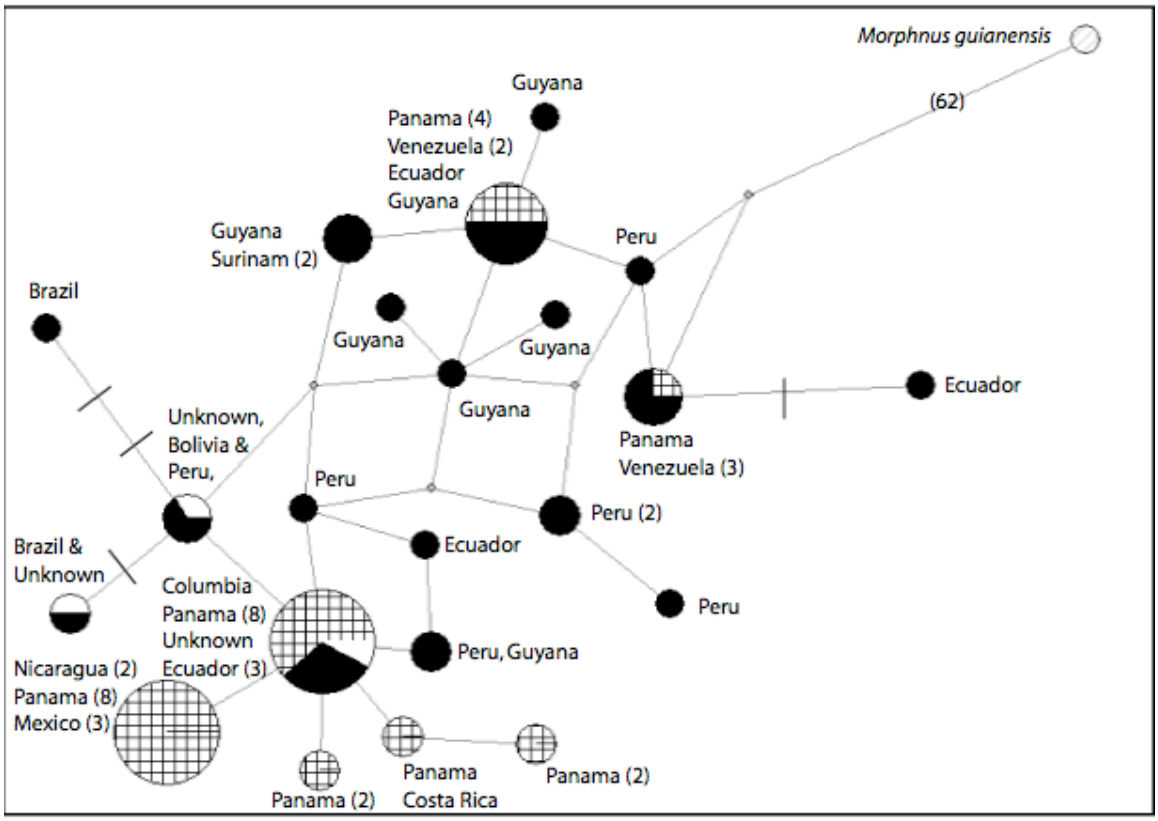
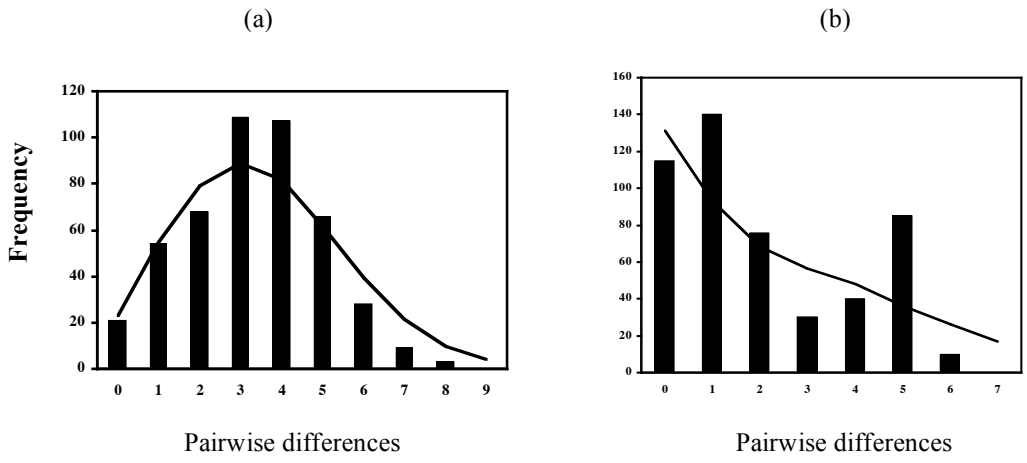


Figure 7. Mismatch distribution for haplotypes found in harpy eagle samples from (a) South America and (b) Central America. The expected distributions of pairwise differences among haplotypes under a model of sudden expansion are shown as lines and the observed distances are shown as bars.



The Nicaraguan and Mexican samples all shared the same haplotype (also found in eight Panamanian birds) and the haplotype of the Costa Rican individual was shared by one Panamanian individual. Haplotypes recovered in South American individuals are found throughout the network with no obvious clusters.

Population demographic histories.—The shape of the observed mismatch distribution for South America is a unimodal curve often found in populations that have experienced a sudden expansion (Rogers, Harpending, 1992), and it was not possible to reject a model of sudden expansion (SSD = 0.0047, p = 0.063, Figure 7a). The low value of Harpending's raggedness index (r = 0.03) reflects the unimodality of the mismatch distribution and is characteristic of an expanding population although it was not significant (p = 0.084). Expansion of the South American region was supported by

significant ($p < 0.05$) negative values of Fu and Li's D^F and F and Fu's Fs (Table 11). Within South America, significant values of D^F, F and Fs were also found in two of the three subgroups: Ecuador-Peru-Colombia and Venezuela-Surinam-Guyana. Values for Tajima's D (not shown) were negative for all groups with the exception of Colombia-Ecuador-Peru, but were also not significant for any of the groups.The estimated time of expansion calculated from τ for South American harpy eagles is apprxominately 60 000 BP (99 000 – 36 000 BP 95% CI).

The mismatch distribution for Central America shows a large number of haplotypes that are identical or that differ by only one nucleotide (Figure 7b.) This shape is associated with populations that have experienced a bottleneck or a very recent expansion (Johnson *et al.*, 2007). A model of population expansion was also not rejected for Central America (SSD = 0.023, p = 0.596, Fig. 2b), and the raggedness index was low (r = 0.060) and not significantly different than expected by chance ($p > 0.713$). In contrast, however, population expansion in Central America was not supported by Fu and Li's D^F and F and Fu's Fs ($p > 0.05$, Table 11).

Genealogy .—The ML topology (not show) resulting from an unconstrained analysis recovered two main clades with low support (bootstrap values of 56 and 60) that did not correspond to geographical origin and a third clade comprised of three Peruvian haplotypes each represented by a single bird (bootstrap value 94). While the constraint tree had a higher likelihood score, the difference in likelihood scores between the unconstrained and constrained phylogenies was not significant ($p>0.10$).

Table 11. Sequence characteristics from 417 bp of mitochondrial domain I control region sequence.

geographic region	individuals	variable sites/ haplotypes	Gene diversity[1], *h*, ± SD	Nucleotide diversity[2], π, ± SD	D^{F3}	F^3	Fs^4
All samples[5]	66	21/23	0.9058 ± 0.020	0.00763 ± 0.0045		-	
Central America	32	9/7	0.7681 ± 0.053	0.00518 ± 0.0033	1.07, n.s.	0.94, n.s.	-0.23, n.s.
Costa Rica-Nicaragua-Mexico	6	2/2	0.3333 ± 0.22	0.001667 ± 0.0017			0.95, n.s.
Panama	26	9/7	0.8031 ± 0.047	0.00567 ± 0.0036			-0.31, n.s.
South America	31	17/18	0.9548 ± 0.018	0.00823 ± 0.0048	-2.59**	-2.55**	-10.20**
Colombia-Ecuador-Peru	14	9/10	0.9231 ± 0.060	0.00764 ± 0.0047			-4.51*
Venezuela-Surinam-Guyana	14	9/8	0.9011 ± 0.052	0.00599 ± 0.0039			-2.68**
Brazil-Bolivia	6	5/3	0.7333 ± 0.16	0.00517 ± 0.0039			1.08, n.s.

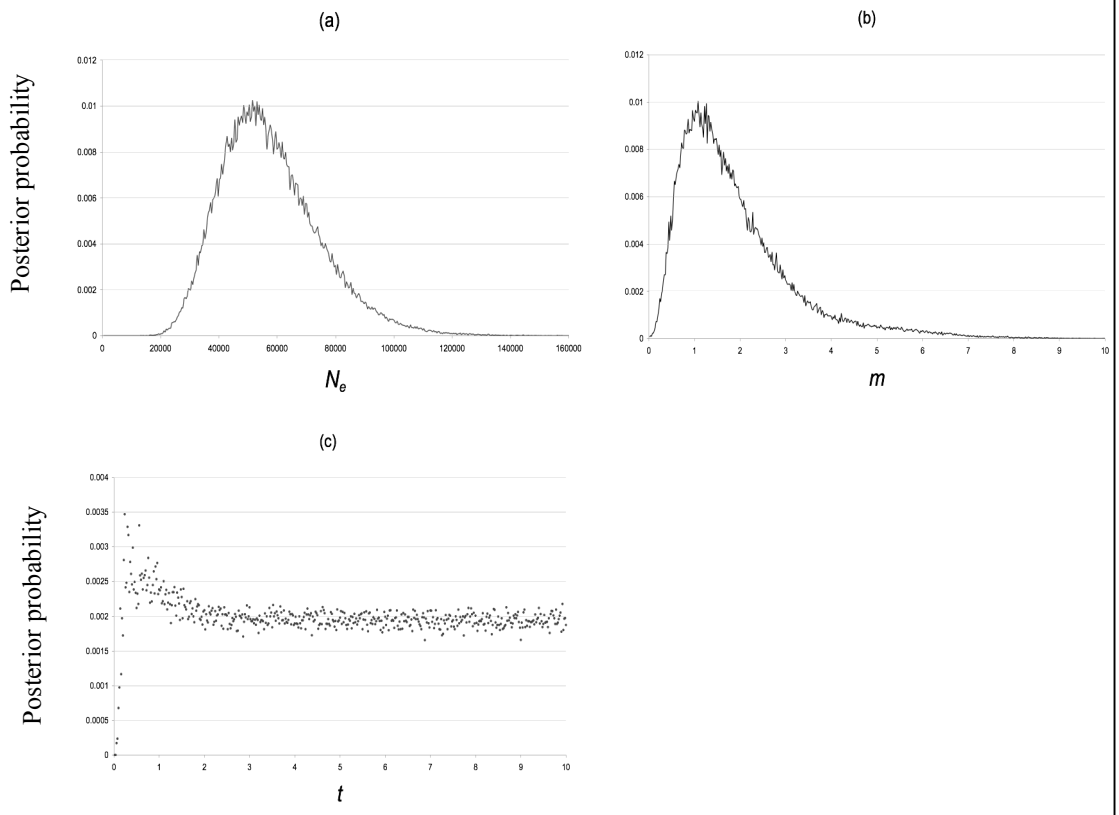
[1]Nei 1987
[2]Tajima 1983
[3]Fu and Li 1993
[4]Fu 1997
[5]includes three samples with unknown geographic localities
* $p<0.05$
** $p<0.01$
n.s= not significant

Coalescent analyses of demography .—The average maximum likelihood estimate of θ_{CA} from three MIGRATE analyses (0.0034, 90% CI 0.00216-0.0060) was an order of magnitude lower than the corresponding average of the South American region (0.040, 90% CI 0.018-0.47). These values correspond to female effective population sizes of 9,406 (4,362-16,804 90% CI) for Central America and 111,787 (51,910-1,300,445 90%) for South America. Likelihood ratio tests rejected the null hypothesis of symmetric migration ($p<0.001$). Higher rates of female gene flow from Central America into South America (m_{CA} = 694.27, 95% CI 341.8-1306.9; m_{SA} = 0.000002, 95% CI 0.000001-.0050) were estimated by the haplotype data.

Parameter estimates from coalescent-based analyses in MDIV produced bell-shaped curves with the exception of T which peaked and approached but did not converge to zero in the upper portion of its distribution (Figure 8). The maximum likelihood estimate of the total effective population size of female harpy eagles was 2.78 (2.52-3.04 95% CI), which corresponds to 51,544 female harpy eagles (27,145-89,728 95% CI). In contrast with the high migration levels found with the program MIGRATE, estimates of migration from MDIV were relatively low, 1.08 (0.83-1.33 95% CI). The maximum likelihood estimate of time since divergence (t=0.24) corresponds to 24,700 years (13,000-43,000 when applying low and high mutation rates). Time to most recent common ancestor is a much older date, corresponding to 308,000 years (179,000-490,000 when applying low and high mutation rates respectively).

Figure 8. Marginal Posterior Probability Densities from MDIV Analyses.

Probability densities for (a) population size (q); (b) migration (m); and, (c) time since divergence (t). The x-axes correspond to the prior range of the parameters



Discussion

In this study we investigated levels of genetic diversity and used phylogenetic and coalescent methods to reconstruct the demographic history for the harpy eagle. We were particularly interested in assessing baseline genetic diversity levels and the level of connectivity or gene flow between geographic areas because of the importance of this information for conservation (Newman, Pilson, 1997; Spielman *et al.*, 2004). Similarly, information about gene flow is important, as conservation initiatives must weigh the

importance of preserving not just total area of habitat, but also connectivity of habitat in the face of increasing fragmentation.

Population structure and demographic history .—There was no evidence of complete barriers to historical gene flow from phylogenetic, coalescent and network analyses, which is consistent with a lack of observed morphological variation across the broad distribution of harpy eagles (Ferguson-Lees, Christie, 2001). We did find evidence for incomplete isolation between geographic regions and among some subgroups with F_{ST} and AMOVA calculations. Significant genetic differentiation (F_{ST} = 0.23, p<0.001) between Central and South America reflects some restriction of gene flow across the Andean mountains as seen with other top predators of Neotropical forests, including the jaguar (Eizirik *et al.*, 2001), ocelot, margay (Eizirik *et al.*, 1998) and puma (Culver *et al.*, 2000). Haplotypes shared between the two major regions were found in close geographic proximity; that is, Panamanian birds shared haplotypes with individuals from northwestern South America.

Lack of monophyly in mitochondrial DNA for birds from the Central and South American regions and divergence time estimates from coalescent analyses suggest that they are separated by a recent partial barrier. The estimate of the divergence time between Central and South America must be interpreted cautiously, however, as the posterior distribution for *t* did not converge upon zero in the upper part of its range. Furthermore, control region mutation rates are highly variable across avian taxa (Ruokonen, Kvist, 2002) and a rate has not been calibrated specifically for Accipitridae taxa. In comparison with other avian taxa, two accipitrid vulture species were found to

have reduced variability in domain I of the control region potentially resulting from a lower mutation rate (Roques, 2004). If the mutation rate of the control region in harpy eagles is lower than that used here, the estimates of divergence time and time to most recent common ancestor would be older than what we calculated. Further sampling of individuals and locales may improve estimates in future work.

Significant F_{ST} values among subgroups within South America and among Central and South American subgroups suggest a pattern of isolation by distance. Increasingly higher values were observed between more distant subgroups and the lowest values were observed between neighboring subgroups. Although our sampling within northern Central America is not sufficient to evaluate the level of connectivity or isolation of more northern areas, it should be noted that there were no haplotypes unique to Central American locations outside of Panama. Every haplotype sampled in Central America was also found in at least one individual from Panama, and two haplotypes were found only in Panamanian birds. Population substructure within Central America has been found in other top predators (e.g., Eizirik *et al.*, 1998; Eizirik *et al.*, 2001) so it is not unlikely for harpy eagles to show some differentiation in Central America. Provided that additional samples could be obtained, future work on harpy eagles should investigate the potential for phylogeographic structure within Central America as this information is important for conservation.

Given the smaller overall size of Central America compared to South America, it is not surprising that coalescent-based analyses estimated a much smaller population size in Central America (i.e. an order of magnitude smaller). Given the extent of recent habitat loss (FAO, 2006), it is likely that harpy eagles in Central America have experienced a

recent bottleneck. The null hypothesis of population demographic expansion was not rejected based on the mismatch distribution (i.e. SSD and raggedness index) for Central America, but these statistics are conservative and use little information in the data (Felsenstein, 1992). Detecting population demographic size changes can be difficult with small sample sizes, few segregating sites or haplotypes, or when the population has experienced a very recent expansion (Ramos-Onsins, Rozas, 2002). Fu's *Fs* has been shown to be more powerful in detecting demographic changes under a variety of conditions including both very recent and older population expansions (Fu, 1997; Ramos-Onsins, Rozas, 2002) and did not support expansion for Central America. Fu and Li's *F* and D^F use an outgroup sequence to identify recent intraspecific mutations and are thus less affected by sample size than test statistics based on mismatch distributions or Fu's *Fs* (Ramos-Onsins, Rozas, 2002). Neither Fu and Li's *F* nor D^F supported expansion, with significant values. While this conflict in measures could reflect a bias in sampling (i.e. sampling over a wide time period or predominantly sampling in the Darien, the evidence for demographic expansion in Central America from our data is weak and it is more likely that the population is at equilibrium or has experienced a very recent bottleneck.

Log-likelihood ratio tests in MIGRATE showed that gene flow is directional with migration from Central into South America and essentially no reciprocal migration. If real, this directional migration could be a result of greater loss and fragmentation of habitat in northern Central America forcing birds into South America. Since our sampling within Central America was densest in the area closest to South America (the Darien of Panama), we should have detected South American migrants to the Darien, but we did not find evidence for migration in this direction.

Within South America there was strong evidence of a recent population expansion. The estimated date of expansion, 60 000 BP (99 000 – 36 000 BP 95% CI), falls entirely within the last ice age and more specifically, well before the last glacial maximum (LGM) of 22 000 – 19 500 BP (Seltzer *et al.*, 2002) . Since the estimated time of expansion, changes in temperature and rainfall in the Amazon basin have been associated with a decrease of rain forest and cloud forest habitat until the LGM (Mayle *et al.*, 2004a) followed by expansion of these habitats to the present time. An increase in deciduous and semi-deciduous forest in the southern Amazon and grassland habitat surrounding the Amazon basin seen during the LGM is proposed to reoccur (Mayle *et al.*, 2004b) coincident with current rapid global climate change involving an increase of *ca.* 3° C and a reduction of annual precipitation of ~20% (Houghton *et al.*, 2001). Given that harpy eagles are found only rarely in dry forests (but see Muñiz-Lopez *et al.*, 2007) and population expansion for harpy eagles is associated with a substantially cooler and wetter time period, anticipated climate and habitat changes present further challenges for the persistence of this species.

Genetic diversity and conservation implications.—High levels of genetic diversity with respect to other Accipitridae species (Table 12) were recovered from the mitochondrial D-loop for 66 harpy eagles. While the inclusion of some older samples could have inflated the genetic diversity measures, we think this effect was minimal for haplotype diversity as only three haplotypes were found exclusively in samples collected before 1960 (two samples from Peru and one from Brazil). It is more likely that our genetic diversity estimates are low for harpy eagles and that additional sampling, both in South

America where most haplotypes sampled were represented by only one or two individuals and in unsampled parts of Central America, would identify more haplotypes and higher genetic diversity.

Higher haplotype diversity among samples from the South American region as opposed to Central America, likely reflects both the larger area of lowland rainforest habitat and larger population size in South America. However, the greater loss of habitat in Central America and the restriction of our sampling to predominantly Panamanian samples likely also plays a part in the lower levels of genetic diversity found for that region (26 Panamanian samples out of 32 Central American samples).

In order to better interpret the amount of genetic variability found for the overall harpy eagle population, it is useful to compare these results with patterns of diversity found in related species (Milot *et al.*, 2007). With respect to mitochondrial control region sequence data published for eight other taxa in the same avian family (i.e. Accipitridae, Table 12) we find high gene diversity and a moderate level of nucleotide diversity for harpy eagles in the DNA sequence we sampled. This suggests that present population impacts may not have reduced levels of genetic diversity in the overall harpy eagle population beyond that which could be aided by conservation efforts. However, the majority of the genetic diversity observed was represented in South American individuals so reduction of genetic variation in Central America is possible and remains a concern.

The lack of major population subdivision and evidence for recent and incomplete isolation among and within regions are consistent with a pattern of gene flow across the broad distribution of harpy eagles. Evidence for statistically significant geographic differentiation supports reduced but ongoing levels of gene flow between

Central and South America and among northern, southern and north-western regions within South America. Thus, attention should be paid to preventing further fragmentation and isolation of harpy eagle subgroups. Active management may indeed be necessary to promote gene flow among isolated remnant populations, particularly in Central America where fragmentation of habitat is greatest and levels of genetic diversity are lowest.

Table 12. Genetic diversity of the control region reported in published studies of Accipitridae taxa.

Species (n=sample size)	IUCN[1] status	Control region domain	Total sites/ Variable sites/%	Haplotypes	Gene diversity, *h* (SD)	Nucleotide diversity, π (SD)
Aquila adalbarti[6] (n=60)	VU	Domain I	345/2/ 0.6	3	0.3215 (0.0730)	0.00098 (0.00024)
Aquila heliaca[6] (n=34)	VU	Domain I	345/8/ 2.3	7	0.7790 (0.0420)	0.00548 (0.00068)
Buteo galapagoensis[2] (n=122)	VU	Domain I	415/5/ 1.2	5	0.625 (0.025)	0.0019
***Harpia harpyja* (n=66)**	**NT**	**Domain I**	**400/21/ 5.3**	**23**	**0.9058 (0.0201)**	**0.007626 (0.004459)**
Milvus milvus[7] (n=105)	NT	Domain I	357/10/ 2.8	10	0.61	0.0032
Buteo swainsoni[2] (n=18)	LC	Domain I	415/18/ 4.3	12	0.766 (0.081)	0.0059
Gypaetus barbatus[3] (n=172)	LC	Domain I	228/28/ 12.3	50	0.932 (0.012)	0.0292 (0.0153)
Haliaeetus leucogaster[4] (n=128)	LC	390 bp Domain I, 163 bp Domain II	553/15/ 2.7	15	0.3497 (0.05447)	0.000806 (0.0008)
Hieraaetus fasciatus[5] (n=72)	LC	Domain I	253/3/ 1.2	4	0.542 (0.046)	0.0024 (0.0017)

[1]World conservation union red list status, vulnerable (VU), near-threatened (NT), least concern (LC, IUCN, 2006)
[2](Bollmer *et al.*, 2005)
[3](Godoy *et al.*, 2004)
[4](Shephard *et al.*, 2005)
[5](Cadahía *et al.*, 2007)
[6](Martinez-Cruz *et al.*, 2004)
[7](Roques, Negro, 2005)

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Chapter 5

Conclusion

The dissertation research presented here assessed phylogenetic relationships for birds of prey in the avian family Accipitridae using molecular sequence from two mitochondrial genes (1047 bases ND2 and 1041 bases cyt-b) and one nuclear intron (1074 bases Beta-fibrinogen intron 7). Relationships among all 14 previously described Accipitridae subfamilies and within four subfamilies of eagles (booted eagles, sea eagles, harpy eagles and snake eagles), two subfamilies of Old World vultures (Gypaetinae and Aegypiinae) and one subfamily of hawks (Buteoninae) were specifically addressed. Monophyly of sea eagles (Haliaeetinae) and booted eagles (Aquilinae) was supported; however, harpy eagles (Harpiinae), snake eagles (Circaetinae), Old World vultures, hawks (Buteoninae) and kites (Milvinae, Perninae and Elaninae) were found to be non-monophyletic. Non-monophyly was also found for the polytypic genera *Aquila*, *Hieraaetus*, *Spizaetus*, *Haliaeetus*, *Leucopternis*, *Buteogallus*, *Buteo*, *Circaetus* and *Accipiter*.

The phylogenies described here highlight multiple examples of convergent evolution throughout the Accipitridae family. Many of these convergences have misled morphological studies and led to non-phylogenetic taxonomy throughout the family. Two examples from this research involve the harpy eagle group and the gymnogene and crane-hawk:

1. Birds of the Harpy eagle group are some of the largest birds of prey, matched in size only by the Condors and two of the sea eagles. The four species described as members of the Harpy Eagle group inhabit primary tropical forest and prey on medium-sized mammals (e.g. monkeys, sloths, tree kangaroos). Two of the species are Old World in distribution, the Philippine eagle (*Pithecophaga jefferyi*) and the New Guinea Harpy Eagle (*Harpyopsis novaeguinea*) and two are of the New World, the Harpy eagle (*Harpia harpyja*) and the Crested eagle (*Morphnus guianensis*). From the phylogeny presented in chapter two it is clear that the Philippine eagle is not closely related to a clade containing the other three harpy eagles. Thus, the harpy eagle lifestyle and associated morphological traits have arisen at least twice independently within the Accipitridae.
2. The Gymnogene (*Polyboroides typus*) has a suite of morphological characteristics related to preying of young birds in cavity nests. These traits include an extended circular range of motion of the tarsus, a short outer toe and a weaker bill. Similar traits are exhibited by the South American crane-hawk (*Geranospiza caerulescens*), a species that also preys on nestlings. These two species are not closely related in our analyses, presenting an example of convergent evolution for specialized limb morphology enabling predation on cavity nesting species.

In this work, reciprocal monophyly and genetic distances of 2% and greater for mitochondrial data characterize nominal sister species of Accipitridae vultures and booted eagles. In chapter two, investigation of subspecies within *Hieraaetus fasciatus* and *H. morphnoides* revealed significant genetic differentiation (7-10% and 2.7-5.8%, respectively) or non-monophyly supporting recognition of *H. spilogaster* and *H. weiskei*

as distinctive species. Based on mitochondrial data in chapter three, the four subspecies of White Hawk (*L. albicollis*) were not monophyletic: *L. a. albicollis* forms a clade with *L. polionotus*, while *L. a. costaricensis, L. a. ghiesbreghti,* and *L. a. williaminae* form a clade with *L. occidentalis*. *L. occidentalis* is currently recognized as a species of conservation due to dwindling numbers and a restricted distribution. Although lack of monophyly for this taxon was found, its geographic distance from other closely related taxa suggests that it may currently be isolated from gene flow. Thus, our results may reflect incomplete lineage sorting in a group undergoing processes that may lead to speciation. Further study is needed to evaluate conflicting hypotheses of incomplete lineage sorting and recent gene flow as they have implications for the species status of this taxon.

Moderate to high levels of genetic diversity were found for the harpy eagle (*Harpia harpyja*) based on 417 bases of the mitochondrial control region from 66 harpy eagles (chapter four). No strong geographical structure was observed with phylogenetic, coalescent and network analyses. However, measurable genetic differentiation was observed between Central and South America and among subgroups within South America suggesting that geographical barriers such as the Andean mountains and Darien straits between northern South America and southern Central America have restricted historical gene flowin the harpy eagle. The estimate of female effective population size for harpy eagles from the Central American region was an order of magnitude smaller than that of South American harpy eagles, likely reflecting both the smaller habitat area available in Central America and the greater recent loss of habitat. Harpy eagles are considered endangered or extinct throughout much of Central America, but are likely to

have experienced less of a detrimental impact in more remote areas of South America. Higher levels of genetic diversity and a recent population expansion were supported for harpy eagles of South American origin. Asymmetric gene flow from Central America into South America suggests that habitat reduction and fragmentation may have driven Central American birds into southern habitats. The results from this work support conservation strategies for harpy eagles that maintain gene flow between southern Central America and South America by preserving connectivity of the mature forest habitat required by these eagles.