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Molecular Phylogenetics and Evolution 35 (2005) 147–164
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

A multi-gene phylogeny of aquiline eagles (Aves: Accipitriformes) reveals extensive paraphyly at the genus level

Andreas J. Helbig[a,*], Annett Kocum[a], Ingrid Seibold[a], Michael J. Braun[b]

[a] *Institute of Zoology, University of Greifswald, Vogelwarte Hiddensee, D-18565 Kloster, Germany*
[b] *Department of Zoology, National Museum of Natural History, Smithsonian Institution, 4210 Silver Hill Rd., Suitland, MD 20746, USA*

Received 19 March 2004; revised 21 September 2004
Available online 24 December 2004

Abstract

The phylogeny of the tribe Aquilini (eagles with fully feathered tarsi) was investigated using 4.2 kb of DNA sequence of one mitochondrial (cyt *b*) and three nuclear loci (RAG-1 coding region, LDH intron 3, and adenylate-kinase intron 5). Phylogenetic signal was highly congruent and complementary between mtDNA and nuclear genes. In addition to single-nucleotide variation, shared deletions in nuclear introns supported one basal and two peripheral clades within the Aquilini. Monophyly of the Aquilini relative to other birds of prey was confirmed. However, all polytypic genera within the tribe, *Spizaetus*, *Aquila*, *Hieraaetus*, turned out to be non-monophyletic. Old World *Spizaetus* and *Stephanoaetus* together appear to be the sister group of the rest of the Aquilini. *Spizastur melanoleucus* and *Oroaetus isidori* are nested among the New World *Spizaetus* species and should be merged with that genus. The Old World *'Spizaetus'* species should be assigned to the genus *Nisaetus* (Hodgson, 1836). The sister species of the two spotted eagles (*Aquila clanga* and *Aquila pomarina*) is the African Long-crested Eagle (*Lophaetus occipitalis*). *Hieraaetus fasciatus/spilogaster* are closest to *Aquila verreauxii* and should be merged with that genus. Wahlberg's Eagle *H. wahlbergi*, formerly placed in *Aquila*, is part of a clade including three small *Hieraaetus* species (*pennatus*, *ayresii*, and *morphnoides*). The Martial Eagle (*Polemaetus bellicosus*) is the sister species of the *Aquila/Hieraaetus/Lophaetus* clade. Basal relationships within this clade remained unresolved. Parsimony reconstruction of the evolution of plumage pattern within Aquilini suggests that: (1) transverse barring of parts of the body plumage was lost in the Palearctic *Aquila–Hieraaetus* clade, (2) pale underparts in adult plumage evolved three times independently, and (3) dimorphic adult plumage is a derived character of the small-bodied *Hieraaetus* clade.

Keywords: Diurnal birds of prey; Accipitriformes; Systematics; Cytochrome *b*; RAG-1; Nuclear introns; Plumage evolution; Biogeography

1. Introduction

Among the hawk-like birds of prey (Aves: Accipitriformes) the tribe Aquilini has long been recognized as a putatively monophyletic subgroup (Amadon and Bull, 1988; Brown and Amadon, 1968; Jollie, 1976–1977). The nine genera belonging to this tribe (*Aquila*, *Hieraaetus*, *Lophaetus*, *Spizaetus*, *Spizastur*, *Stephanoaetus*, *Polemaetus*, *Oroaetus*, and *Ictinaetus*; for a full taxonomic list see Appendix) differ from most other birds of prey in that their tarsometatarsus is fully feathered down to the toes, as opposed to being naked and scaled as in most other raptors. Referring to this character, Amadon (1982) suggested the common name "booted eagles" for the tribe. Monophyly of group as a whole is poorly supported by phenotypic characters (feathered tarsi also occur in other, clearly unrelated, species such as Rough-legged Hawk *Buteo lagopus*, Snowy Owl *Bubo scandiacus*) and was contradicted by a preliminary cladistic analysis of 188 osteological characters (Holdaway, 1994). Relationships among major lineages within the

* Corresponding author. Fax: +49 38 30050441.
E-mail address: helbig@uni-greifswald.de (A.J. Helbig).

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doi:10.1016/j.ympev.2004.10.003

group have also remained largely unknown. Here we use DNA sequences of one mitochondrial and three nuclear genes to address questions about the relationships of the Aquilini both at the phylogenetic and the molecular level.

At the phylogenetic level we were interested in: (1) whether the Aquilini as defined by Amadon (1982) are monophyletic and (2) whether genera within the tribe represent monophyletic groups of species. Possible affinities of the large Neotropical eagles *Harpia* and *Morphnus* with booted eagles have repeatedly been suggested (e.g., Amadon, 1982). We therefore included these species in our study. Although their tarsi are not feathered, the juvenile plumage in both of these genera strikingly resembles that of *Stephanoaetus*, *Polemaetus*, *Oroaetus*, and some *Spizaetus* in being largely white below. Monophyly of each of the larger aquiline genera *Aquila* (11 species), *Hieraaetus* (7 species), and *Spizaetus* (10 species) is doubtful. Among the suggested generic characters, none appear to be strong synapomorphies. For instance, *Aquila* species are distinguished from other eagles by proportionately larger bill with a relatively straight proximal part of the culmen, lack of occipital crest, uniformly dark plumage with no barring of body plumage, and immature plumages being similar to those of adults (Amadon, 1982). However, none of these characters is unique to *Aquila*. Differences of *Hieraaetus* to other aquiline genera are all gradual, no discrete characters were listed by Amadon (1982). The African Wahlberg's Eagle (*wahlbergi*) has usually been included in *Aquila* (Amadon, 1982; Brown and Amadon, 1968; Sibley and Monroe, 1990; Stresemann and Amadon, 1979), although its plumage dimorphisms (pale and dark-morph adult) and whistling vocalizations are very atypical of that genus (Brown et al., 1982). It was recently shifted to *Hieraaetus* by Ferguson-Lees and Christie (2001) without explicit justification.

Common features of *Spizaetus*, the only aquiline genus except *Aguila* to be distributed both in the New World (2 species) and the Old World (8 species), also seem weakly informative phylogenetically: nares round (elliptical in *Hieraaetus*), adult body plumage usually barred or streaked (in parts) and quite different from juvenile, occipital crest of 3–5 elongated feathers usually present. Among the monotypic genera, *Polemaetus* has been united with *Hieraeetus*, *Stephanoaetus*, and *Lophaetus* with *Spizaetus*, while *Spizastur* has been suggested to be derived from the Neotropical *Spizaetus* lineage (Amadon, 1982), thus making this genus paraphyletic. Brooke et al. (1972) suggested that the African Long-crested Eagle *Lophaetus occipitalis* is closely related to *Aquila*, making generic separation doubtful. However, none of these suggestions was based on a formal phylogenetic analysis.

Plumage patterns have played a major role in the taxonomy of eagles, but their phylogenetic utility is unknown. We therefore used the molecular phylogeny to investigate plumage evolution. Adult plumages of aquiline eagles can be categorized as follows:

(1) pied pattern: dark upperparts, light underparts with spots or longitudinal blotches, but without transverse barring of body plumage,
(2) pied pattern with some *transverse barring* on underparts (may be reduced to thighs),
(3) largely dark body plumage lacking any transverse barring (note that flight feathers [remiges and rectrices] are often paler or whitish from below and show dark transverse bars in many species).

Species with pattern (1) are either monomorphic for the pied plumage, or dimorphic with a pied and an all dark (brown or rufous) morph. This results in four easily recognizable adult plumage categories, which were mapped onto the phylogeny using parsimony criteria.

The extensive sequence data set assembled here for eagles lends itself to further investigate a number of questions at the molecular level: (1) To what extent are phylogeny estimates derived from mitochondrial and nuclear gene sequences congruent? (2) Do introns of autosomal genes contain qualitative molecular characters (insertions, deletions) that are phylogenetically informative? and (3) Do these qualitative characters support the same relationships as does the "quantitative" single-nucleotide variation? Previous studies in birds have used introns of the β-fibrinogen (Prychitko and Moore, 2003; Weibel and Moore, 2002) and myoglobin gene (e.g., Ericson et al., 2003), both of which were found to be phylogenetically quite informative at the intergeneric level. In the hope of broadening the spectrum of phylogenetically useful introns we used two for which previous experience is more limited: intron 5 of the adenylate-kinase gene (Shapiro and Dumbacher, 2001) and intron 3 of the lactate dehydrogenase gene (Friesen et al., 1999).

2. Materials and methods

2.1. Taxon sampling

This study is part of a broader effort to understand the phylogenetic relationships of birds of prey. As outgroup taxa we used the Secretary Bird *Sagittarius serpentarius*, the putative sister species of Accipitridae (Sibley and Ahlquist, 1990), and the Osprey *Pandion haliaetus*, previously shown to occupy a very basal position within, and probably representing the sister lineage of, Accipitridae (Seibold and Helbig, 1995a; Sibley and Ahlquist, 1990; Wink and Sauer-Gürth, 2000). Bearded Vulture *Gypaetus barbatus*, Harpy Eagle *Harpia harpyja*, and Crested Eagle *Morphnus guianensis* were used as

further outgroup taxa successively more closely related to the Aquilini. Taxon sampling of the ingroup comprised 25 species, one of which was represented by two subspecies (*Aquila chrysaetos*).

Blood, tissue or feather samples were acquired from bird parks, zoos, and museum tissue collections (Table 1). For most species sequences were verified from samples of at least two specimens acquired from independent sources. Most birds were sampled when still alive, so voucher material usually consists of original blood/feather samples plus photographic documentation and in some cases entire or partial specimens.

Table 1
Geographic origin and museum collection numbers of samples used in this study

Species	Coll. No.	Origin
Sagittarius serpentarius (Secretary bird)	ZMUG RA01S321	South Africa
	ZMUG RA01S001	Walsrode Birdpark (captive)
Pandion h. haliaetus (Osprey)	ZMUG RA02H749	Germany
	ZMUG RA02H34	Germany
Gypaetus barbatus (Bearded Vulture)	TPB WAA1D993	Mongolia (captive-bred)
	TPB WAA1D994	Mongolia (captive-bred)
Harpia harpyja (Harpy Eagle)	TPB W276	not known (Zoo Nürnberg)
	TPB W277	not known (Zoo Nürnberg)
Morphnus guianensis (Crested Eagle)	ZMUG RA35G34	Venezuela
Aquila audax (Wedge-tailed Eagle)	ZMUG RA05A49	Australia (TPB)
Aquila chrysaetos chrysaetos (Golden Eagle)	ZMUG RA05C15	Switzerland
	ZMUG RA05C23	Switzerland
Aquila chrysaetos canadensis (Golden Eagle)	ROM 1B-2350	Canada
Aquila heliaca (Imperial Eagle)	ZMUG RA05H01	Marlow Birdpark (captive)
	ZMUG RA05H759	Israel
Aquila nipalensis (Steppe Eagle)	ZMUG RA05N11	Israel
	ZMUG RA05N16	Kazakhstan
Aquila rapax (Tawny Eagle)	ZMUG RA05R64	South Africa
	ZMUG RA05R40	Zimbabwe
Aquila pomarina (Lesser Spotted Eagle)	ZMUG RA05P13	Germany
	ZMUG RA05P440	Germany
Aquila clanga (Greater Spotted Eagle)	ZMUG RA05C755	Israel
	ZMUG RA05C16	Belarus
Aquila verreauxii (Verreaux's Eagle)	ZMUG RA05V51	South Africa
	ZMUG RA05V52	South Africa
Hieraaetus wahlbergi (Wahlberg's Eagle)	TPB 217707	South Africa
	ZMUG RA05W04	South Africa
Hieraaetus ayresii (Ayer's Hawk-Eagle)	ZMUG RA26A138	South Africa
Hieraaetus morphnoides (Little Eagle)	MVM 2209	Victoria, Australia
Hieraaetus pennatus (Booted Eagle)	ZMUG RA26P129	Germany
	ZMUG RA26P226	Israel
Hieraaetus fasciatus (Bonelli's Eagle)	TPB 9208, 9984	Morocco
	ZMUG RA26F422	Israel
Hieraaetus spilogaster (African Hawk Eagle)	ZMUG RA26S311	Zimbabwe
	ZMUG RA26SB1	South Africa
Lophaetus occipitalis (Long-crested Eagle)	ZMUG RA31O193	South Africa
	ZMUG RA31O194	South Africa
Oroaetus isidori (Black-and-Chestnut Eagle)	ZMUG RA44.1	Colombia (captive*)
Spizaetus tyrannus (Black Hawk Eagle)	TPB 4F7193	Not known (Antwerp Zoo)
Spizaetus ornatus (Ornate Hawk Eagle)	ZMB 1998/006	Not known
Spizaetus cirrhatus (Changeable Hawk Eagle)	ROM 1B34	Not known
	BMNH S/2002.45.1	Not known (London Zoo)
Spizaetus nipalensis (Mountain Hawk Eagle)	BMNH S/2002.44.1	Not known (London Zoo)
Spizaetus philippinensis (Philippine Hawk Eagle)	ZMUC 113933	Isabella, Philippines
Spizastur melanoleucus (Black and White Hawk Eagle)	ZMUG RA45M1	Colombia
	USNM 616782	Guyana
Stephanoaetus coronatus (Crowned Eagle)	ZMUG RA46C62	Zimbabwe
	ZMUG RA46C02	South Africa

Collections: BMNH—British Museum Natural History, Tring, UK; MVM—Museum Victoria, Melbourne, Australia; ROM—Royal Ontario Museum, Toronto, Canada; USNM—US National Museum, Smithsonian Institution, Washington DC; TPB—Tierpark Berlin, Germany; ZMB—Naturkundemuseum Berlin, Humboldt University; ZMUC—Zoological Museum University of Copenhagen, Denmark; ZMUG—Zoological Museum University of Greifswald, Germany.

Notes. *C. Marquez Raptor Facility, Bogota. The cyt *b* sequence of *Aquila adalberti* was taken from Seibold et al. (1996).

2.2. Choice of loci

To resolve both relatively recently evolved and more ancient relationships, we chose loci with quite different rates of evolution and different kinds of variation. Partial sequences were obtained of the nuclear RAG-1 gene (1978 bp) that had previously been used in "deep" phylogenetics of birds (Barker et al., 2001; Groth and Barrowclough, 1999). Two introns of nuclear genes, intron 5 of the adenylate-kinase gene (AK-5; Shapiro and Dumbacher, 2001) and intron 3 of the lactate dehydrogenase gene (LDH-3; Friesen et al., 1999) were included, in particular to look for potentially informative insertion/deletion variation. Finally, we extended the sequence matrix of the mitochondrial cytochrome *b* (cyt *b*) gene, which we had previously used to reconstruct relationships of aquiline eagles (Seibold et al., 1996) and other raptors (Seibold and Helbig, 1995a,b, 1996), to the full length of 1143 nucleotides.

2.3. Laboratory methods

Total cellular DNA was isolated by proteinase K digestion (overnight at 60 °C) followed by a salting-out procedure (Miller et al., 1988). Amplification and sequencing primers were designed from published sequences and optimized for application in birds of prey (Table 2). For each of the four loci, we developed several sets of amplification primers for use in alternative reactions, thereby increasing the chances of successful amplification. This strategy also allowed us to check for congruence between sequences derived from the same target DNA but amplified by different primer combinations. The mitochondrial cyt *b* gene was sequenced from at least two overlapping long-fragment PCRs in order to avoid coamplification of possible nuclear copies. Primers were located either in the tRNAs flanking the ND5 and cyt *b* genes (tRNA-Leu–mt-F, product length ca. 2400 bp), or near the 3′-end of the ND5 gene and in the 12sRNA gene (ND5–DDL). The latter primer combination amplified the cyt *b* and ND6 genes plus the entire control region. Primers for amplification and sequencing of nuclear introns were located in flanking exon regions. Published exon sequences were aligned and conserved regions 60–80 nucleotides upstream and downstream of the intron, respectively, were chosen as priming sites. Amplification primers for the nuclear RAG-1 gene were taken from Groth and Barrowclough (1999).

PCRs were run on Perkin–Elmer (GeneAmp 2400) and Biometra (Primus 96) thermocyclers at the highest possible annealing temperatures. The Expand Long template PCR System (Boehringer–Mannheim) was used for long-fragment amplifications of mitochondrial DNA according to the manufacturer's specifications. Excess

Table 2
Sequences of amplification and sequencing primers

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Cyt *b* (cds)	*Amplification*	
	tLeu-AYCTTGGTGCAAATCCAAGT	DDL-GCACCGCCAAGTCCTTAGAGTT
	ND5-ACCTACCTAGGAWAYTTYGC	mt-F-TCTTCAGTTTTTGGTTTACAAGAC
	Sequencing	
	mt-C-TGAGGMCARATATCCTTCTGAGG	mt-B-CCTCAGAAGGATATYTGKCCTCA
	mt-D-GAYAAAATCCCATTYCAYCC	mt-E-GGGTTTGCTGGGGTRAARTTTTC
RAG-1 (cds)	*Amplification*	
	RAG-13-TCTGAATGGAAATTCAAGCTGTT	RAG-R22-GAATGTTCTCAGGATGCCTCCCAT
	RAG-F2-CTCAGCACCAAGCTGCTTGCAGTTGA	RAG-R6-CCATGTCCTTTAAGGCASAAACCA
	Sequencing	
	S4-GCCAGATCTGTGAGCATATT	S3-CARTTGTGACAAAATRGAGTGGG
	S5-GATATAAAGGCTGTATGCATGAC	FL1-GGGAAGCAAGGATACCAGCAGG
	FL2-TGCCTTAAAGGACATGGAGGA	
LDH (intron 3)	*Amplification*	
	B1-CAAACTAAAAGGAGAAATGATGGA	NP10-CACATTCCTCTGAACCAGGTTGAG
	LDH-F2-ATGATGGATCTACAGCATGG	B4-GGGCTGTATTTNACRATCTGAGG
	Sequencing	
	B1′-CTAAAAGGAGAAATGATGGA	B2-TTCCTCTGAACCAGGTTGAG
AK (intron 5)	*Amplification*	
	AK1-GACACGGTGYTGGACATGCT	AK6f-CGCTTCTTGATGGTCTCCTC
	AK5b-GACGGCTACCCCCGCGAGGTG	AK6c-TCCACCCGCCCRCTGGTCTC
	Sequencing	
	AKA2-CTGCGGGAYGCCATGGTGGCC	AK6g-CGCTTCAGCAGGCGTTTCACC
	AK5c-GAGAGGAGTTTGAGAARAAGG	

Forward (sense strand) and reverse primers were used in various combinations to achieve optimal amplification and check for sequence congruence between amplifications.

amplification primers and nucleotides were digested with Exonuclease I (10 U) and Shrimp Alkaline Phosphatase (2 U; PCR product pre-sequencing kit, Amersham). Cycle sequencing reactions were performed with Ampli-Cycle Sequencing Kit (Perkin–Elmer), either with [α-^{33}P]dATP for manual sequencing or with fluorescent labelled primers for detection in a Li-cor 4200 sequencer.

2.4. Phylogenetic analysis

Sequences were aligned by eye, which was unproblematic not only for the two coding genes, but also for most of the two introns, which contained several insertions and deletions ("indels" hereafter) relative to the outgroup sequences. Each indel of more than two nucleotides was regarded as a single mutational event, irrespective of its length (as recommended by Simmons and Ochoterena, 2000). To see whether such indels supported the same branches as did single-nucleotide variation, phylogenetic trees were constructed from sequence matrices in which alignment gaps were coded as missing data. Informative indels were then mapped a posteriori onto the trees using parsimony criteria (see Figs. 2–4).

Each gene was tested for homogeneity of base frequencies using the chi-square test in PAUP* 4.0b10 (Swofford, 2003). Trees were constructed from single-locus or concatenated sequence matrices using four methods: (1) neighbor-joining (NJ) with Kimura 2-parameter distance (MEGA 2.0; Kumar et al., 2001); (2) maximum likelihood using the quartet puzzling (QP) algorithm (TREE-PUZZLE 5.2; Schmidt et al., 2004) with the HKY85 model (Hasegawa et al., 1985); (3) maximum likelihood (ML) using heuristic searches performed with PAUP*; (4) Bayesian analysis (program MrBayes, Huelsenbeck and Ronquist, 2001; http://morphbank.ebc.uu.se/mrbayes/) using the General Time Reversible (GTR) substitution model with Γ-distributed rate variation. Other phylogenetic algorithms (minimum evolution, maximum parsimony with various weighting schemes) generally yielded similar topologies and bootstrap values (data not shown).

Branch support was assessed by bootstrapping (neighbor-joining; 1000 replicates), quartet puzzling (10,000 steps), and MCMC (Markov Chain Monte Carlo) methods (Bayesian analysis). For each Bayesian analysis, four Markov chains of 2,000,000 generations were run to estimate posterior probabilities. Trees were sampled every 500 generations and the first 200,000 generations were discarded as burn-in. Thus, each resulting consensus tree with posterior branch probabilities was based on 3600 sampled trees. For concatenated sequence matrices, model parameters were estimated separately for each of the three data partitions (cyt *b*, RAG-1, introns; likelihood settings: Nst = 6, rates = invgamma). The 'covarion' (variable rate of evolution of a site over time) and 'variable omega' models (variable non-synonymous/synonymous rate ratio across codons) were applied to the coding sequence partitions.

For ML heuristic searches with PAUP*, models of sequence evolution and rate heterogeneity parameters were evaluated using methodologies described in ModelTest version 3.06 (Posada and Crandall, 1998). First, an NJ tree was produced using Jukes–Cantor distances. Parameters were then calculated for 56 nested models of sequence evolution on the NJ tree and the models evaluated with ModelTest using a hierarchical likelihood ratio test and the successive approximations approach suggested by Swofford et al. (1996). The selected model was then applied to heuristic ML tree searches performed with PAUP*. The original data were analysed with TBR branch-swapping and 10 random addition searches. In addition, 100 bootstrap pseudoreplicate datasets were analysed with TBR branch-swapping and one random addition search per run. Branches with less than 50% support are generally shown as unresolved polytomies.

Because of the very different rates of evolution and mutational biases between nuclear and mitochondrial genes, we explored the possibility of phylogenetic incongruence among loci before combining data (Bull et al., 1993; Swofford et al., 1996). Data partitions were regarded as being in conflict if they supported incongruent clades of the same taxa with bootstrap proportions of >75% or Bayesian posterior probabilities of >95%. To test whether the degree of incongruence among the actual loci in the data set was significantly different from random partitions of the same data, we used the incongruence length difference (ILD) test of Farris et al. (1995) as implemented in PAUP*, where it is known as the partition homogeneity test.

Some hypotheses of relationship were examined by comparing globally optimal trees to the best trees that could be found compatible with each hypothesis. Hypotheses examined included monophyly of traditional genera, traditional genera expanded with conservative additions of taxa, and a biogeographic hypothesis. In each analysis, a particular group was constrained to be monophyletic in PAUP*, and a heuristic ML search was conducted to find the optimal tree under that constraint, using the same model of sequence evolution as the unconstrained search. The difference in log likelihood of the constrained tree versus the unconstrained tree was taken as a measure of support for a particular hypothesis or its alternative.

To obtain age estimates for the divergence events within the Aquilini, we calculated two kinds of genetic distances: (1) from cytochrome *b* sequences, Kimura 2-parameter distance with Γ-correction ($\alpha = 0.2$, estimated using TREE-PUZZLE); a rate of 1.6% sequence divergence per 1 million years was calculated assuming a fossil-based age of 17 million years for modern Buteonine hawks (Olson, 1985) and using the split between *Haliae-*

etus and *Buteo* lineages (sequences from Seibold and Helbig, 1996) as representative of that radiation; (2) from RAG-1 sequences: uncorrected divergence; rate: 0.056% per million years (based on RAG divergences reported by Groth and Barrowclough (1999) and an age of "Ciconiiform" orders of about 74 million years, van Tuinen and Hedges (2001)). Using the *Haliaeetus–Buteo* split at 17 million years yields a very similar RAG divergence rate of 0.058% per million years (unpublished sequences; Helbig et al. (in prep.)). RAG-1 sequences have been shown previously to be unsaturated even for large genetic divergences (birds vs. mammals; Groth and Barrowclough, 1999) so the divergence rate can be assumed to be linearly related to time. A likelihood ratio test did not reject a molecular clock within Aquilini either with the cyt *b* or the nuclear sequences, so the assumption of a uniform rate of molecular evolution within the ingroup is valid. Although a large error may be associated with clock calibrations for these genes, the estimates do indicate relative age differences between clades and rough absolute ages.

Sequences were deposited in the EMBL nucleotide sequence data bank. Accession numbers are as follows: cytochrome *b* gene—AJ604483–AJ604511, AJ812238, Z73463; RAG-1 gene—AJ601440–AJ601467, AJ812239; LDH intron 3—AJ601497–AJ601525, AJ812241; AK-1—intron 5/exon 6: AJ601468–AJ601496, AJ812240.

3. Results

3.1. Sequence variation across four loci

The alignment of the two coding genes, complete cytochrome *b* (1143 bp) and partial RAG-1 (1978 bp, corresponding to position nos. 84–2073 of the published chicken sequence; M58530; Carlson et al., 1991), did not require the insertion of gaps. Note, however, that this region of the chicken RAG-1 gene contains a 15-bp insertion and a 3-bp deletion relative to Accipitriformes (Groth and Barrowclough, 1999). Alignment of the two nuclear introns required the insertion of several indels, which varied in length from 1 to 17 nucleotides. The total length (including gaps) of the AK intron 5 alignment for all Aquilini plus outgroup taxa was 545 bp, which includes at the 3′-end 72 bp of the flanking exon 6 sequence (corresponding to positions 5382–5453 of the published chicken sequence; D00251, Suminami et al., 1988). The alignment of LDH intron 3 was 556-bp long and contained no flanking exon sequence.

Tests for homogeneity of base frequencies at variable sites were performed for each gene separately. No evidence of heterogeneity among taxa was detected. However, nucleotide composition and ts/tv ratios (as estimated by TREE-PUZZLE) varied widely between the four genes (Table 3). Cyt *b* was relatively high in A + T (51.6%) and low in G (12.5%), as is often the case for mtDNA sequences. RAG-1 and LDH-3 were also AT-rich (55.7 and 63.1%, respectively), while AK-5 was CG-rich (A + T = 37.1%). As expected, the levels of sequence divergence for nuclear loci were much lower than for the mitochondrial cyt *b* gene, and the RAG-1 coding gene was more conservative than the two introns (Table 3). The entire nuclear data set of slightly over 3.0 kb provided 130 potentially informative sites (4.2%), whereas the cyt *b* gene at 1.1 kb provided 360 (31.5%) informative sites.

LDH intron 3 contained two deletions (4 bp each) informative within the ingroup, plus an autapomorphic 3-bp deletion in *Stephanoaetus coronatus*. AK intron 5 contained a 17-bp deletion informative within the ingroup and a 3-bp deletion shared by aquiline eagles plus *Harpia*/*Morphnus* relative to the other outgroup taxa. In addition, the AK intron 5 included near its 5′-end a tandem repeat region consisting of 3–7 copies of the motif TGCCA or CGCCA. Most aquiline eagles

Table 3
Characterization of sequence variation in the four genes studied

Parameter	Cyt *b*	RAG-1	LDH-3	AK-5
Type of sequence[a]	cds	cds	Intron	Intron
Length of alignment (nc)[b]	1143	1978	556	545[d]
Variable sites (all taxa/Aquilini only)[c]	463/377	179/66	95/32	107/27
Informative sites (all taxa/Aquilini only)[c]	360/284	65/24	33/16	32/16
Estimated ts/tv ratio (all taxa/Aquilini only)	3.90/6.39	2.91/5.72	3.28/4.89	3.79/2.90
Max. sequence divergence (%, uncorrected)[c]				
Sagittarius vs. all others	15.40	4.58	8.63	9.35
Within Aquilini	10.85	1.22	2.16	2.59
Nucleotide composition (%)	A 27.2	A 32.2	A 28.8	A 19.7
	T 24.4	T 23.5	T 34.3	T 17.4
	C 35.9	C 21.0	C 15.0	C 34.2
	G 12.5	G 23.3	G 22.0	G 28.7

[a] cds, coding sequence.
[b] Including gaps.
[c] Nucleotide variation only (gaps not counted as fifth character state).
[d] Includes 72 nc exon sequence at 3′ end.

possessed 4 such units, but *Spizaetus cirrhatus* had 5 and *S. philippensis* had 6. The outgroup taxa had 2 (*Pandion*), 3 (*Harpyia*, *Morphnus*), 6 (*Sagittarius*), and 7 (*Gypaetus*) units, respectively. The complete intron alignments are available from the EMBL-Align database (Accession Nos. LDH: ALIGN_000751; AK: ALIGN_000752).

To study the level of saturation of mitochondrial relative to nuclear sequences, we plotted the numbers of transitions (ts) and transversions (tv) in the cytochrome *b* against the total divergence of the concatenated nuclear data set (Fig. 1). Cyt *b* transitions are clearly saturated for ingroup versus outgroup comparisons, but little saturation is evident within the ingroup. Transversion differences appear unsaturated across the entire range of divergences.

3.2. Tree reconstruction and congruence between data partitions

In a first step, all four data partitions were analysed separately. Each of the four genes supported monophyly of the Aquilini (as far as they were represented in this study). This was true both with the sampling of outgroup taxa presented here, and for a much larger sampling of accipitriform species of 49 genera (Helbig et al., in prep.). Individually, the three nuclear-encoded genes yielded good support for only few clades. This was due to the small number of informative sites in each nuclear gene (Table 3). Although the 50% bootstrap consensus trees were not fully congruent between the three data partitions, incongruent clades were never supported by bootstrap values over 70% (Fig. 2). The partition homogeneity (or ILD) test also found no incongruence between any of the four genes, including cyt *b*. We therefore combined sequences of the three genes into a "nuclear concatenated" (NC) data set with a total length of 3079 nucleotides (including alignment gaps).

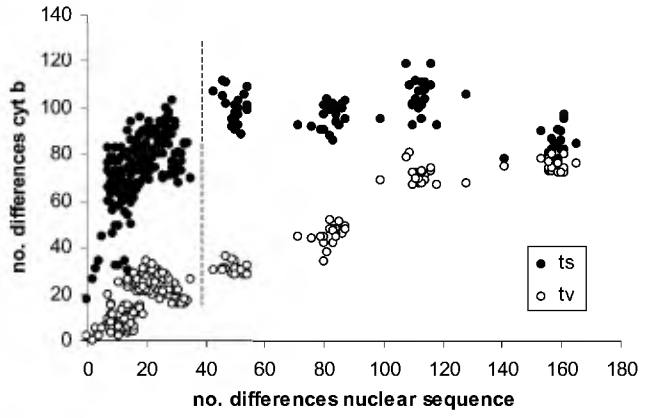

Fig. 1. Numbers of pairwise nucleotide differences in cytochrome *b* (ts and tv separately) vs. total number of differences in nuclear concatenated sequences (alignment gaps were not counted). Symbols to the left of the broken line refer to species pairs within the Aquilini.

Phylogenetic signal was congruent or complementary between (a) nuclear and mitochondrial sequences as well as between (b) single-nucleotide variation and indel variation in the NC data set (Fig. 3). The NC data set yielded better resolution for basal branches than did cyt *b* sequences. Cyt *b* sequences, on the other hand, resolved some relationships between closely related species with higher support, but were uninformative with regard to the basal branching pattern within Aquilini and within the *Aquila–Hieraaetus* group. Inclusion of additional genera of accipitriform raptors did not alter the phylogeny of the ingroup (results not shown).

Three deletions in the LDH and AK introns were found to be potentially informative within the ingroup, a fourth united *Harpia* and *Morphnus* with Aquilini to the exclusion of the three other outgroup taxa. One, a 17-bp deletion of the AK intron (indel 2, Fig. 3), was shared by all ingroup species except Old World *Spizaetus* and *Stephanoaetus*. This deletion, therefore, unambiguously characterized the larger of the two basal clades within Aquilini. In addition, this clade was strongly supported by single-nucleotide variation of the concatenated nuclear sequence matrix (Fig. 3). Two other deletions, both 4 bp in length and located in the LDH intron 3, were shared by *Hieraaetus fasciatus/spilogaster* and six *Aquila* species (Figs. 2 and 3). However, neither of the two nested clades indicated by these 4-bp deletions was strongly supported in the bootstrap consensus trees based on either mitochondrial or nuclear single-nucleotide variation. In fact, the clade sharing indel 4 excludes *Aquila chrysaetos* and thus conflicts with relationships indicated by cytochrome *b*, which firmly places *chrysaetos/canadensis* as sister to a clade containing *A. verreauxii* and others. This case of nuclear–mitochondrial conflict may indicate that indel 4 is homoplasious, i.e., the deletion was regained in *A. chrysaetos*.

3.3. Comparisons of alternative hypotheses

The three traditional genera *Spizaetus*, *Aquila*, *Hieraaetus* appeared non-monophyletic in all our trees. To see whether the sequence data strongly contradicted monophyly of these genera, we compared the likelihoods of various data partitions under alternative constrained topologies to that under the optimal topology (Table 4), using a difference in log likelihood units of two or more as an indication of substantial difference in likelihood support (Edwards, 1992). Monophyly of *Spizaetus* requires a decrease of 46 or more log likelihood units for each data partition. Even if *Spizastur melanoleucos* and *Oroaetus isidori* are allowed to form a clade with *Spizaetus*, monophyly requires a substantial decrease in likelihood for nuclear and combined data sets. Similarly, monophyly of both *Aquila* and *Hieraaetus* implies substantially reduced likelihood for all data partitions. A less stringent hypothesis that includes *Hieraaetus fascia-*

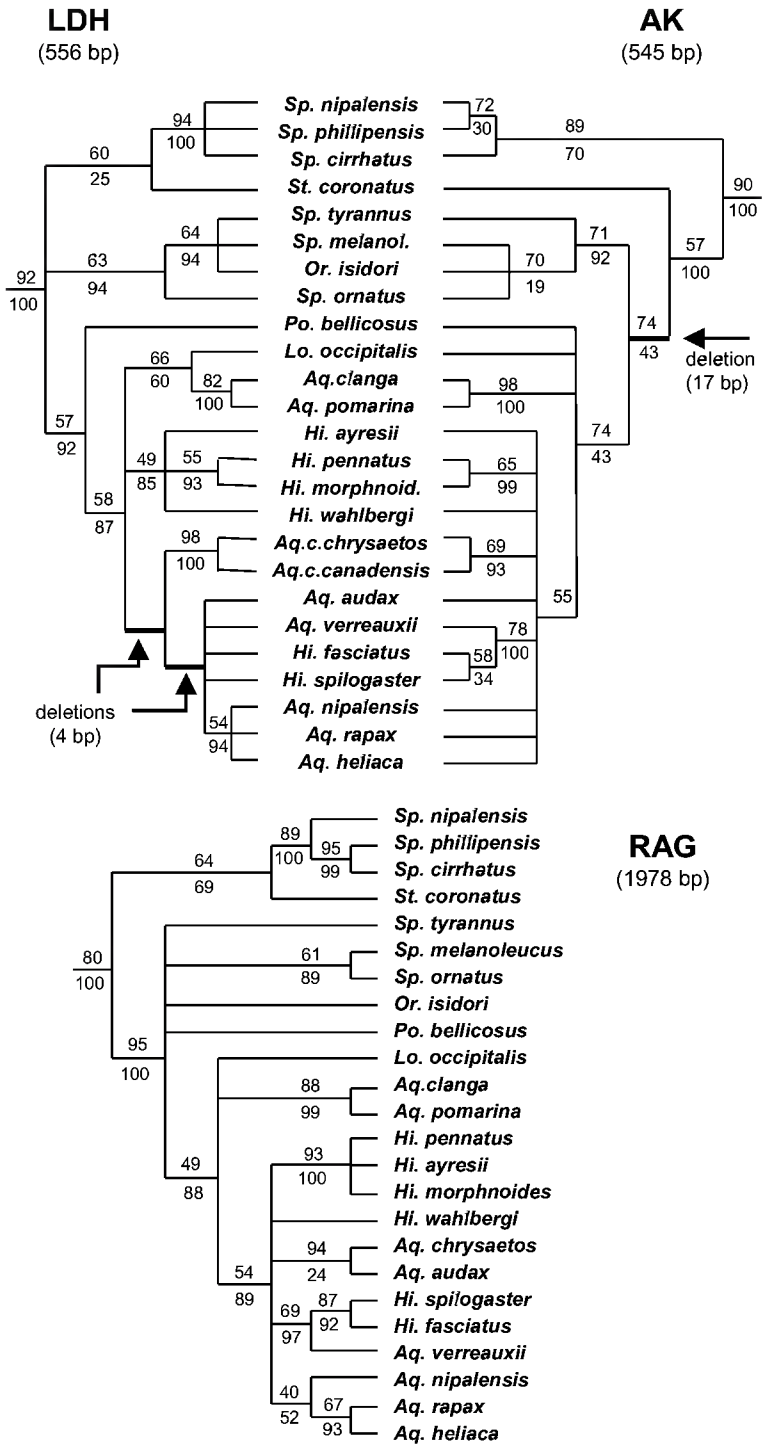

Fig. 2. Phylogeny of aquiline eagles based on sequences of two nuclear introns (top) and RAG-1 coding region (bottom). Consensus trees derived by neighbor-joining (Kimura 2-P distance, 1000 replicates; above branch) and Bayesian analysis (GTR model, 3600 trees, below branch) are shown in which branches are drawn as resolved only if they received >50% support in at least one of the analyses. Placement of outgroups was identical for all loci (cf. Fig. 3).

tus/spilogaster with a monophyletic *Aquila* still requires a sizeable decrease in likelihood for all data sets.

Another obvious hypothesis to be examined is that Neotropical and Palearctic species within the Aquilini form mutually monophyletic clades. This is not strongly contradicted by the cyt *b* data, but is by the nuclear loci (Table 4) and by a 17-bp deletion (AK intron) that the four Neotropical species share with all other Aquilini except *Stephanoaetus* and the Palearctic *Spizaetus* (indel 2, Fig. 3).

The fact that mitochondrial and nuclear data partitions did not completely agree in the hypotheses they

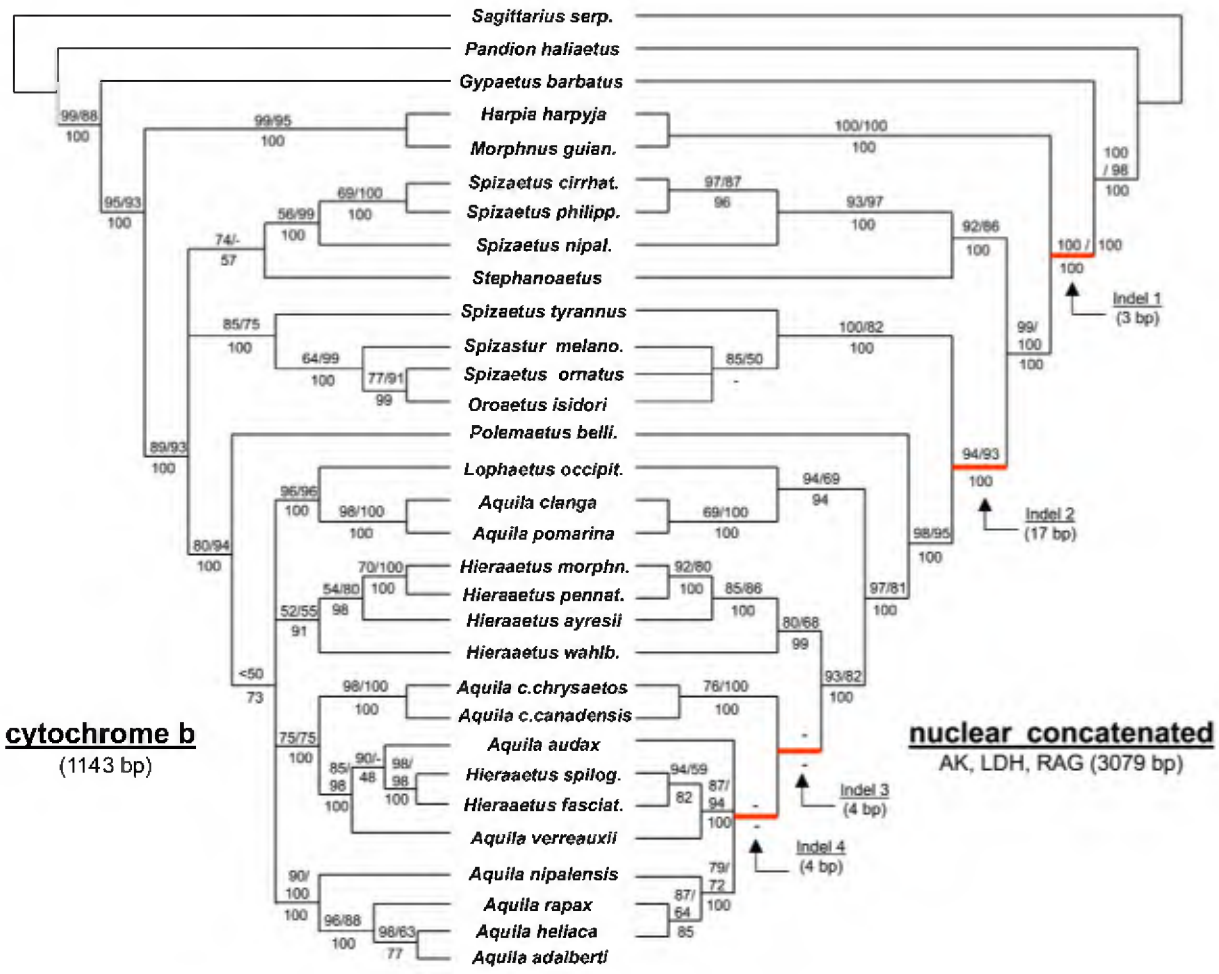

Fig. 3. Phylogeny of aquiline eagles derived from mitochondrial cytochrome *b* sequences (left) and the nuclear concatenated data set (genes as in Fig. 2, right). Branches supported by intronic insertions/deletions are shown in bold. Both topologies represent consensus trees using criteria as in Fig. 2. Numbers are quartet puzzling/ML support values (10,000 steps/100 replicates; above branch) and Bayesian posterior probabilities (below branch). Branches with less than 50% support were collapsed except when supported by deletions (–). The parameter values for the models of sequence evolution used in the ML bootstraps are given in Table 5.

support or reject shows that phylogenetic signal of the two data partitions is complementary in the sense that some clades are significantly supported by both partitions, others are supported by one partition but not by the other.

3.4. Phylogenetic inferences

Because the nuclear and mtDNA partitions are largely complementary in signal, and appear to have little, if any, significant conflict, we wished to combine them for final ML bootstrap and Bayesian analyses. However, base composition and sequence divergence differed markedly among genes (Table 3), so estimated models of sequence evolution are quite different for the nuclear and mtDNA partitions (Table 5). The nuclear gene model has fewer parameters, relatively even base frequencies, a mild excess of transitions over transversions (~3.5 to 1), and moderate among site rate variation modeled by a single parameter (Γ-distributed rates). The cytochrome *b* model has strongly skewed base composition, a great excess of transitions over transversions (>12 to 1), and two parameters to account for more extreme among site rate variation (Γ-distribution and a proportion of sites invariant). Nevertheless, separate ML tree searches on the two partitions produced similar trees with no strongly conflicting nodes (Fig. 3).

It therefore seemed justified to combine sequences of all four genes into a 'total evidence' data set. This was analysed with MrBayes, applying different models to mitochondrial, nuclear coding and non-coding partitions. Current versions of PAUP* allow only a single model of sequence evolution, and that estimated for the four gene combined data set is something of a hybrid (Table 5). Base composition resembles the three nuclear genes because the total length of nuclear sequence is roughly three times that of mtDNA

Table 4
Comparison of alternative tree topologies

Constrained group	Δln *L*		
	Cyt *b*	Nuclear	Total
Aquila monophyletic[a]	50.82	30.71	82.39
Aquila + *Hi. fasciatus*/*spilogaster* monophyletic[b]	13.46	12.02	25.48
Hieraaetus monophyletic[c]	32.35	18.70	52.61
Spizaetus monophyletic[d]	74.16	46.28	111.84
Spizaetus, *Spizastur*, *Oroaetus* monophyletic[e]	1.58	22.61	17.23
Palearctic & Neotropical species mutually monophyletic[f]	1.01	16.35	13.16

Constrained and unconstrained ML heuristic tree searches were conducted in PAUP* with models of sequence evolution estimated from the various data partitions (Table 5). Each constrained tree was compared to the respective optimal unconstrained ML tree topology for (a) cytochrome *b* (ln $L = -7863.16$), (b) nuclear concatenated (ln $L = -7194.01$) and total concatenated datasets (ln $L = -15{,}754.33$). Numerical values of Δln *L* are the decreases in likelihood of the constrained versus the unconstrained trees.

Genus abbreviations: *Aq—Aquila; Hi—Hieraaetus*, *Sp—Spizaetus*, *Or—Oroaetus*.

[a] (*Aq.clang*, *Aq.poma*, *Aq.heli*, *Aq.rapa*, *Aq.nipa*, *Aq.chry*, *Aq.cana*, *Aq.auda*, *Aq.verr*).

[b] (*Aq.clang*, *Aq.poma*, *Aq.heli*, *Aq.rapa*, *Aq.nipa*, *Aq.chry*, *Aq.cana*, *Aq.auda*, *Aq.verr*, *Hi.fasc*, *Hi.spilo*).

[c] (*Hi.fasc*, *Hi.spilo*, *Hi. penn*, *Hi. morph*, *Hi. ayre*, *Hi. wahl*).

[d] (*Sp. cirr*, *Sp. phil*, *Sp. nipa*, *Sp.tyra*, *Sp. orna*).

[e] (*Sp. cirr*, *Sp. phil*, *Sp. nipa*, *Sp.tyra*, *Sp. orna*, *Spizastur*, *Or.isid*).

[f] (*Aq.clang*, *Aq.poma*, *Aq.heli*, *Aq.rapa*, *Aq.nipa*, *Aq.chry*, *Aq.cana*, *Aq.auda*, *Aq.verr*, *Hi.fasc*, *Hi.spilo*, *Hi. penn*, *Hi. morph*, *Hi. ayre*, *Hi. wahl*, *Sp. cirr*, *Sp. phil*, *Sp. nipa*, *Lophaetus*, *Polemaetus*, *Stephanoaetus*), (*Sp.tyra*, *Sp. orna*, *Spizastur*, *Or.isid*).

Table 5
Models of sequence evolution estimated for various data partitions (see Methods)

	Cytochrome *b* (TVM + I + Γ)	3 Nuclear genes (HKY85 + Γ)	4 Genes combined (GTR + I + Γ)
Base frequencies			
A	0.3071	0.2908	0.2846
C	0.4166	0.2248	0.2781
G	0.1009	0.2381	0.2097
T	0.1754	0.2463	0.2276
Substitution rates			
A↔C	0.6645	1.0000	1.9785
A↔G	12.1121	3.4744	9.9191
A↔T	0.7559	1.0000	1.0225
C↔G	0.2160	1.0000	0.8074
C↔T	12.1121	3.4744	18.1564
G↔T	1.0000	1.0000	1.0000
Γ shape (α)	1.2020	0.2097	0.5554
Proportion of invariant sites (I)	0.5385	0.0000	0.6456

TVM—transversion model (transition rates equal). HKY85—Hasegawa et al. 1985 (transition rates equal, transversion rates equal). GTR—general time reversible (substitution rates symmetrical within types, free to vary between types). Rates are relative to G↔T.

sequence. The substitution rate matrix is dominated by cytochrome *b*, which has many more variable sites. Among site rate variation is intermediate in the combined model, requiring two parameters, but a less extreme α value for the Γ-distribution than that found for cytochrome *b*.

The tree topologies resulting from ML and Bayesian analyses of the 4 gene combined data set (Fig. 4) are identical for all nodes but one, which had weak support in either analysis. Serendipitously, indel 3 is informative for that node, and we show it with the topology that is most parsimonious for that character. The Bayesian posterior probability was greater than 95% for all but two nodes and the ML bootstrap percentages were greater than 95% for all but six nodes. As might be expected, both the topology and support values of nodes on the combined tree tend to reflect the nuclear DNA signal at the base and the mtDNA signal at the tips. Fig. 4 represents our best estimate of the phylogeny of the group, based on all of the available molecular data. The following major phylogenetic inferences can be drawn from these analyses:

(1) Among the aquiline eagles there are three basal clades: (I) Palearctic *Spizaetus* with *Stephanoaetus* as their probable sister species; (II) Neotropical *Spizaetus*, among which are nested *Spizastur melanoleucus* and *Oroaetus isidori*; (III) an Old World clade of *Aquila*, *Hieraaetus*, and *Lophaetus* species with *Polemaetus* as their sister species. These find-

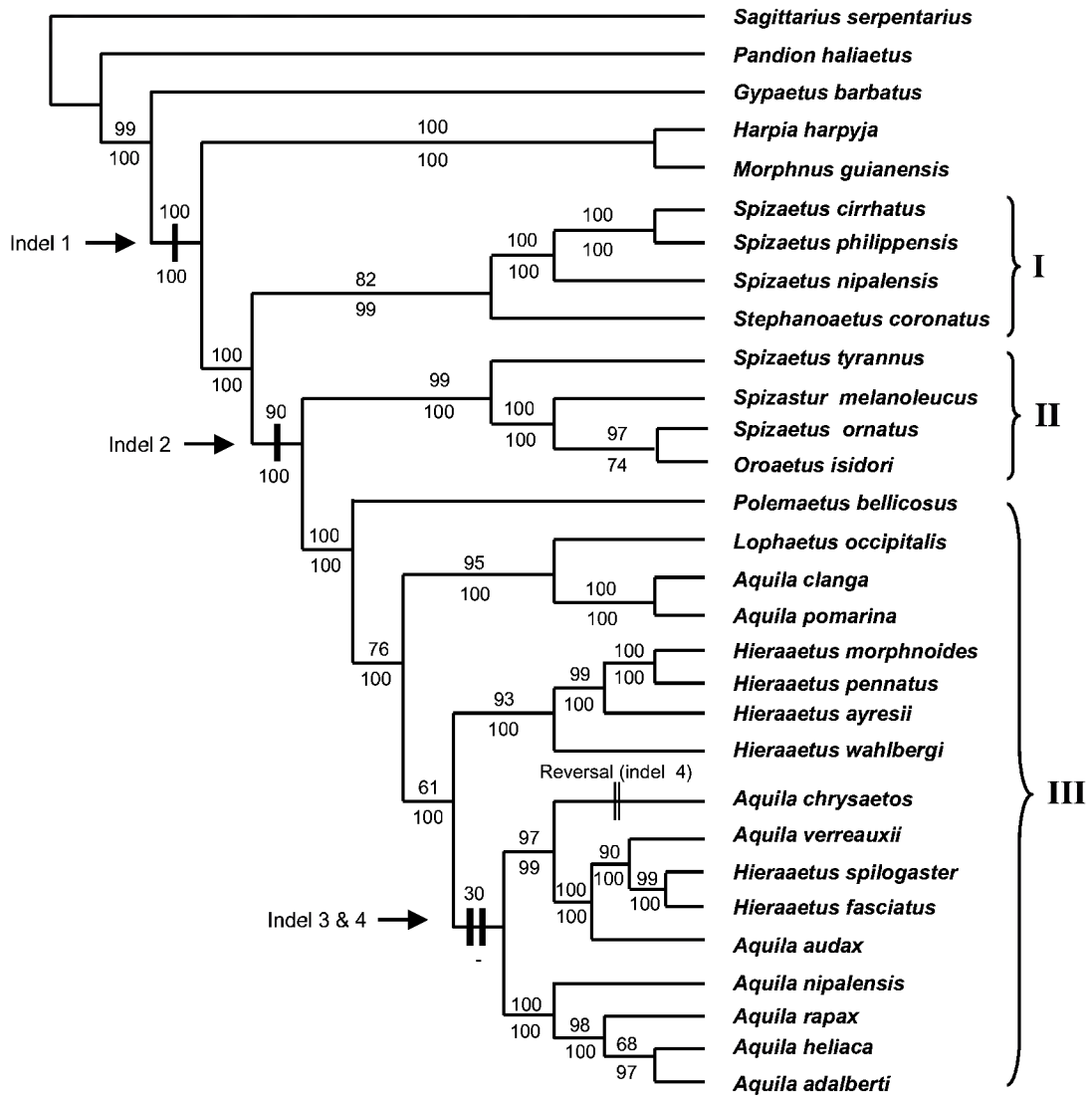

Fig. 4. Phylogeny of aquiline eagles derived from the combined nuclear and mitochondrial DNA data and indel variation. Roman numerals designate major clades (compare text). Numbers are ML bootstrap support values (100 replicates; above branch) and Bayesian posterior probabilities (below branch). The model of sequence evolution for both analyses was general time reversible with a proportion of invariant sites and Γ-distributed among site rate variation. The model parameter values for the ML bootstrap are given in Table 5. Only cytochrome *b* data were available for *Aquila adalberti*, so its indel character states are not known.

ings are supported by both nuclear and mitochondrial sequences (Fig. 3).

(2) The New World clade (II) is the sister group of the Old World clade III as indicated by nucleotide variation of the concatenated nuclear loci and a shared 17-bp deletion in the AK intron (Fig. 3).

(3) All genera of Aquilini that contain more than a single species (*Spizaetus*, *Aquila*, and *Hieraaetus*) are non-monophyletic in their classical delimitation. This is strongly indicated both by nuclear and mitochondrial sequences (Table 4).

(4) Bonelli's Eagle (*H. fasciatus*) and African Hawk Eagle (*H. spilogaster*) are nested within an *Aquila* clade that excludes *A. clanga* and *A. pomarina*. The latter two are most closely related to the African Long-crested Eagle (*Lophaetus occipitalis*).

(5) The smaller *Hieraaetus* species (*pennatus*, *morphnoides*, and *ayresii*) form a monophyletic group with *H. wahlbergi* as their sister.

Among the *Aquila* eagles, sister relationships are strongly supported for *clanga/pomarina*, *chrysaetos/canadensis* (subspecies level) and *heliaca/adalberti* (allospecies level). The latter pair forms a clade with *nipalensis* and *rapax*. Support for more inclusive groups is ambiguous: mtDNA (cyt *b*) shows the Golden Eagle (*chrysaetos/canadensis*) to be sister to a group consisting of *A. verreauxii*, *A. audax*, and *H. fasciatus/spilogaster*, while the distribution of indel 4 in the LDH intron seems to contradict such a relationship, but may be homoplasious (see above). Single-nucleotide variation of the concatenated nuclear loci was not informa-

tive with regard to these fairly recently evolved relationships.

3.5. Evolution of plumage patterns

Plumage patterns were mapped onto the consensus phylogeny using parsimony criteria (Fig. 5). The pied pattern with transverse barring of the adult body plumage found in *Stephanoaetus* and all *Spizaetus* species is also present in *Harpia* (reduced to thighs) and Crested Eagle *Morphnus guianensis*. This appears to be an ancestral character within Aquilini. Within the tribe, transverse barring was apparently lost secondarily in *Spizastur*, *Oroaetus* and in the lineage leading to *Polemaetus*. It is also lacking in the clade comprising the genera *Lophaetus*, *Aquila*, and *Hieraaetus*, which are characterized by an all dark body plumage (in dimorphic species restricted to dark-morph adults), except for *H. fasciatus*/*spilogaster*, whose monomorphic pied pattern appears to be a complete reversal from the all dark plumage seen in their closest relatives. The regular occurrence of two adult morphs (pied and all dark) in the small-bodied *Hieraaetus* species appears to be a derived character restricted to that clade.

The transition from juvenile to adult plumage is another character that seems to have evolved conservatively among Aquilini: all basal lineages (New and Old World *Spizaetus*, *Stephanoaetus*, *Polemaetus*) share with the outgroup (*Harpia*, *Morphnus*) a sharp contrast between a juvenile plumage with whitish, unpatterned underparts versus a much darker adult plumage with heavily patterned underparts. This dichotomy was modified in *Spizastur* and *Spizaetus tyrannus*, but in opposite directions: in *Spizastur* adult plumage resembles the juvenile in retaining unmarked whitish underparts, while in *Spizaetus tyrannus* juvenile plumage is already heavily

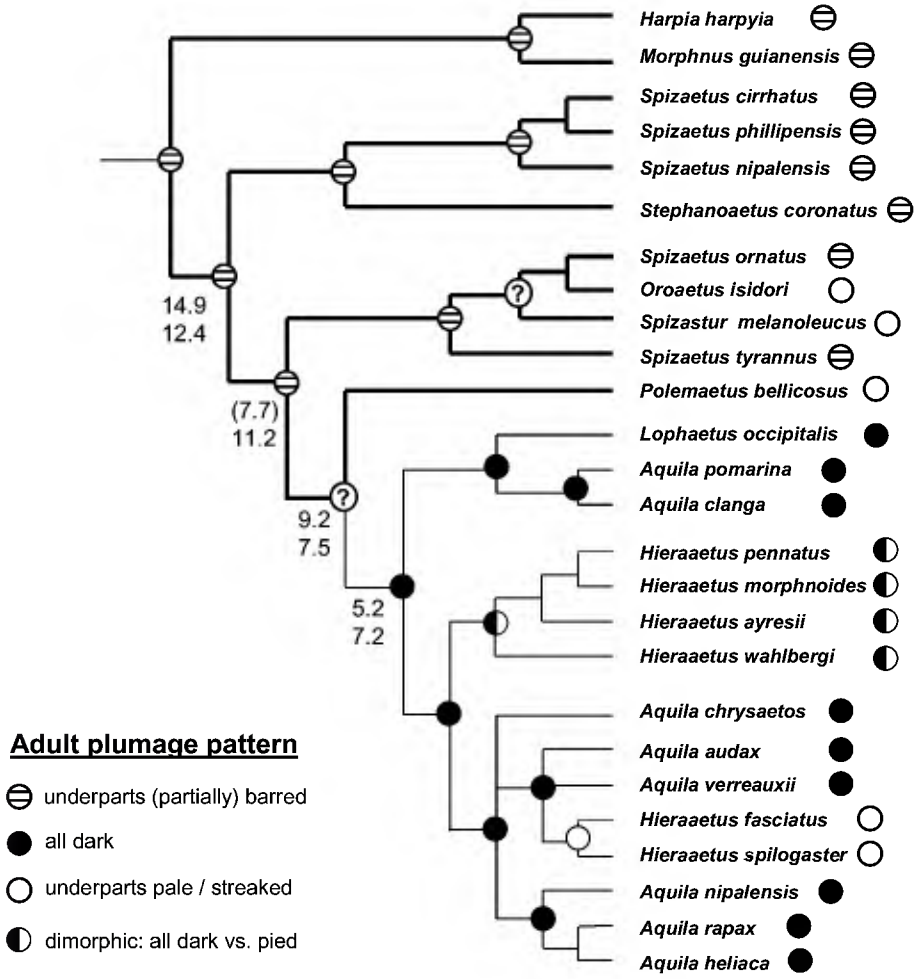

Fig. 5. Plumage patterns of aquiline eagles mapped onto the consensus phylogeny using parsimony. Adult plumage pattern is shown by round symbols. The contrast between juvenile and adult plumage is coded by branch thickness: bold branches, unpatterned juvenile plumage with whitish underparts contrasts with darker, heavily patterned adult plumage; thin branches, juvenile plumage more similar to adult, lacking whitish, unmarked underparts (except pale morph *H. pennatus*, which is very similar to adult). Numbers are age estimates in million years for basal splits derived from RAG-1 (upper) and cyt *b* (lower) divergences. Value in parentheses is probably an underestimate due to apparent rate slowdown of RAG-1 in the Neotropical clade.

marked (as in adults of other species), although still whitish on head and chest.

The strong dichotomy between juvenile and adult plumage was apparently lost in the lineage ancestral to the *Lophaetus–Aquila–Hieraaetus* clade, in which juvenile plumages lack unmarked whitish underparts and, although often being paler, are generally more similar to adults.

3.6. Age estimates

We derived age estimates for clades within the Aquilini and the group as a whole from cyt *b* and RAG sequences (Fig. 5). RAG-based dates for the basal nodes range from 15 million to 5.2 million years; corresponding values based on cyt *b* range from 12.4 million to 7.2 million years. These estimates suggest that extant aquiline lineages date back to about 12–15 million years. The Neotropical clade diverged 8–11 million years ago, the *Polemaetus* lineage diverged between 7.5 and 9 million years ago, while the basal lineages of the genera *Lophaetus*, *Hieraaetus*, and *Aquila* diversified in a period about 5–7 million years ago.

The different spread of age estimates probably reflects the fact that RAG divergence is more linearly related to time, whereas even Γ-corrected values of cyt *b* divergence may not fully overcome the saturation effect inherent in sequences of this fast-evolving gene. On the other hand, RAG-based dates for the most recent splits, which have accumulated few nucleotide differences, may be subject to relatively larger stochastic error.

4. Discussion

The present study is the first comprehensive attempt to reconstruct relationships of aquiline eagles based on a molecular phylogenetic analysis. The combination of relatively conservative nuclear with more rapidly evolving mitochondrial sequences yielded a well-resolved phylogeny. Separate analyses of nuclear and mtDNA data partitions yielded results in which neighbor-joining (NJ), maximum likelihood (ML, quartet puzzling), and Bayesian analysis agreed very well. Bootstrap proportions were quite similar between the first two methods, while posterior probabilities of the latter were often much higher. However, two clades in the cyt *b* tree received distinctly *lower* Bayesian pp than NJ or ML support: (1) the grouping of *Stephanoaetus* with Old World *Spizaetus*, a clade that was strongly supported by nuclear sequences; and (2) the grouping of *Aquila audax* with *Hieraaetus fasciatus/spilogaster*, which is in conflict with the nuclear phylogeny (Fig. 3) and does not occur in the combined tree (Fig. 4). All clades that Bayesian analysis did recover with reasonable probability (>90%) were also identified in the bootstrap consensus phylogenies derived by NJ and ML, albeit sometimes with much lower support. In other words, there was no conflict between Bayesian and other kinds of analyses.

4.1. Nuclear–mitochondrial congruence

There are now several examples of good agreement between phylogenies based on mitochondrial and nuclear sequences in birds (e.g., Birks and Edwards, 2002; Irestedt et al., 2002; Johnson and Clayton, 2000; Prychitko and Moore, 2000). However, at deeper phylogenetic levels, such as the basal relationships of birds and mammals, congruence between mitochondrial and nuclear sequences proved more difficult to recover due to the stronger effects of mutational bias in the mitochondrial genome (Paton et al., 2002; Phillips and Penny, 2003). This varying degree of congruence between mitochondrial and nuclear sequence data means that levels of conflict vs. congruence must be assessed on a case by case basis, because they depend on the phylogenetic level of interest and the particular kind of sequences involved.

In our study, there was not only excellent congruence between nuclear and mitochondrial sequence data sets, but these two basic data partitions also yielded complementary phylogenetic information in that nuclear sequences resolved some basal branches that cyt *b* did not resolve, while cyt *b* resolved some peripheral clades that were poorly resolved by nuclear data. Analyses of the 4 gene combined data set yielded higher nodal support values throughout the tree than either partition alone. This was true even though the model of sequence evolution applied in the combined ML tree searches was significantly different from the optimal one estimated for either data partition. Again, the generally higher support values found in combined analyses suggest that the phylogenetic signals from nuclear and mtDNA partitions are largely congruent.

The only case of apparent nuclear–mitochondrial conflict we identified in our data set concerned a peripheral branch within *Aquila*. The interpretation that indel 4, which is in conflict with the mitochondrial phylogeny (Fig. 3), may be homoplasious is suggested by the fact that the group sharing this indel was not supported by single-nucleotide variation at any of the three nuclear loci nor all three combined. This contrasts with the more complex, 17-bp AK intron deletion (indel 2), which is shared by a large clade within Aquilini that was also strongly supported by nuclear single-nucleotide variation.

4.2. Indels

Indels are *discrete* molecular characters and can serve as strong phylogenetic markers (Lloyd and Calder, 1991). Since indel variation is due to different molecular mechanisms than are point mutations, it is not subject to

the same biases that affect single-nucleotide variation. Indels, therefore, provide an independent source of evidence that can reinforce conclusions derived from nucleotide substitutions. This approach has been successfully applied in diverse organisms (e.g., Bapteste and Philippe, 2002; Kawakita et al., 2003; Venkatesh et al., 2001) including birds (Ericson et al., 2000; Prychitko and Moore, 2003).

Small indels are often the result of varying numbers of direct repeats. Such repetitive elements may lead to length variation due to strand slippage during replication (Nishizawa and Nishizawa, 2002), rendering them prone to homoplasy on phylogenies. However, only one of the four informative indels in our study involved a repetitive element (indel no. 1 was an AGC direct repeat), and it was not homoplasious. Variable-number direct repeats did occur near the 5′-end of the AK intron, but proved not very informative phylogenetically.

Indels in unique, non-coding sequence are less common than substitutions, and seem, a priori, much less likely to be homplasious. In theory, the likelihood of homoplasy should be inversely related to the size of an indel (Vogt, 2002), although no empirical support was found for this expectation when comparing 1- and 2-base gaps with longer ones (Simmons et al., 2001). Among eagles only two of the four groups sharing a particular indel were strongly supported also by single-nucleotide variation (namely those sharing indels 1 and 2, Fig. 3), while one was in conflict with a node strongly supported in both the mtDNA and combined trees (group sharing indel 4, Fig. 3). This shows that it is the mutual support between the two kinds of variation, not necessarily an indel per se, that should be interpreted as strong phylogenetic evidence.

4.3. Phylogeny and taxonomic consequences

Previous attempts at clarifying aquiline relationships (Amadon, 1982; Jollie, 1976–1977) were based on morphological and behavioural similarities, not on character analysis using outgroup comparisons that would have been necessary to clarify polarity of character change. It may thus not be surprising that all genera of aquiline eagles that contain more than a single species turned out to be non-monophyletic. Amadon in his 1982 revision regarded each of these genera as monophyletic, although he conceded that not all species shared the characteristics he regarded as typical of the respective genera.

Recognition of the genus *Spizaetus* has always been poorly founded, based on transverse barring on the underparts (also present in *Stephanoaetus* and outside Aquilini), occipital crest (most species) and round nares. Jollie (1976–77) recognized that New World and Old World *Spizaetus* are not each other's closest relatives and suggested that the former are closer to *Oroaetus*, while the latter are closer to *Stephanoaetus* and *Polemaetus*. Amadon (1982) merged *Stephanoaetus*, *Lophaetus*, and *Oroaetus* with *Spizaetus*, but did not include *Polemaetus*, which he assigned to *Hieraaetus*, or *Spizastur*, which he upheld as a monotypic genus (see also Amadon and Bull, 1988). Our results confirm that the genus *Spizaetus* is polyphyletic, consisting of an Old World and a New World lineage. However, the two New World species are not each other's closest relatives, since *Spizaetus ornatus* is more closely related to *Oroaetus* and *Spizastur* than to the congeneric *S. tyrannus*. All four New World species should, therefore, be united in a single genus '*Spizaetus*' Vieillot 1816. The seven Asian *Spizaetus* species, three of which were subject of this study, are undoubtedly monophyletic. Their sister species appears to be the African Crowned Eagle *Stephanoaetus coronatus*. So the seven Asian '*Spizaetus*' should be assigned to a different genus, for which the name '*Nisaetus*' Hodgson 1836 is available (see Wolters, 1976). Finally, there is the Cassin's Hawk Eagle *Spizaetus africanus* of Africa, which was not included in our study and whose relationships with Asian "*Spizaetus*" appear doubtful (for instance, it lacks the transverse barring of the underparts typical of the Asian species). The generic placement of this species remains to be determined.

There is moderate molecular support for the monophyly of the small-bodied *Hieraaetus* species, four of which were represented in our study (*pennatus*, *ayresii*, *morphnoides*, and *wahlbergi*). These species share two potential phenotypic synapomorphies: (1) dimorphic adult plumage (see below); (2) vocalizations include high-pitched, drawn-out whistles (on territorial display), but lack the repetitive 'clucking, barking, and yelping' territorial calls typical of *Aquila* species, *Stephanoaetus* and *Polemaetus* (Amadon, 1982; Brown et al., 1982). No formal phylogenetic analysis of eagle vocalizations has yet been presented, although vocal characters may be quite informative.

Traditional delimitation of the genus *Aquila* was primarily based on the relatively large bill and a dark plumage with little difference between juvenile and adult, characters that may clearly be plesiomorphic. An unexpected finding of our study was that the Palearctic *Aquila pomarina* and *A. clanga* form a strongly supported monophylum with the Afrotropical Long-crested Eagle *Lophaetus occipitalis*. Together with *A. hastata*, which is close to *pomarina* and *clanga* (Parry et al., 2002), they should thus be merged into the genus *Lophaetus* Kaup 1847. "*Aquila*" *wahlbergi*, which Brooke et al. (1972) believed to be closely related to *A. pomarina*, is very distant from it and should certainly not be included in *Lophaetus*.

Our results indicate that *Hieraaetus fasciatus* and *H. spilogaster* are nested within *Aquila*, among which they appear most closely related to *A. verreauxii*. They should

thus be assigned to the genus *Aquila*. Beyond this strongly supported change, our data neither support nor contradict the monophyly of a genus *Aquila* that would include *fasciatus/spilogaster*, but exclude *pomarina/clanga*. A single 4-bp deletion (indel 3) in the LDH intron is the only evidence in favour of such a group. Given the lack of evidence for any alternative arrangement, we recommend a conservative approach maintaining a large genus *Aquila* (incl. *fasciatus*, *spilogaster*, excl. *clanga*, *pomarina*), although strong evidence for its monophyly is lacking.

The African Tawny Eagle *A. rapax* and the Asian Steppe Eagle *A. nipalensis* have long been regarded as conspecific (Brown and Amadon, 1968; Stresemann and Amadon, 1979; Vaurie, 1965) or members of a superspecies (Amadon, 1982). However, Brooke et al. (1972) and Clark (1992) pointed out that they are quite different in morphology, behaviour and ecology and are certainly differentiated at the species level. In the molecular phylogeny, we find no evidence that the two are even sister species. *A. rapax* is the sister species of the Imperial Eagles *A. heliaca/adalberti* while *nipalensis* is the sister of all three.

4.4. Evolution of plumage patterns

Previous classifications of birds of prey heavily relied, among other characters, on plumage patterns. The lack of knowledge about the likely evolution of plumage patterns among these eagles seems to have been a major reason for the non-phylogenetic taxonomy of this group practised so far. For instance, the idea of a monophyletic genus *Aquila* seems to be based largely on its uniformly dark plumage that differs from the more varied pattern in most other eagles. Our molecular phylogeny offers a means to interpret plumage evolution based on an independently derived tree. Clearly, the evolution of plumage patterns in this group is constrained by phylogeny, i.e., patterns do not vary at random across the tree. This is true both for certain features of the adult plumage and the differentiation between juvenile and adult plumages. The occurrence of transverse barring on the ventral body plumage of adults, although certainly a character that evolved repeatedly among Accipitridae, appears to be plesiomorphic within aquiline eagles. It is thus not suited to support a close relationship of *Stephanoaetus* with *Spizaetus* (Amadon, 1982), although molecular data do support such a relationship with Old World *Spizaetus*. The dimorphic pattern found among adults of the small *Hieraaetus* species, *pennatus*, *ayresii* (dark morph rare) *morphnoides* and *wahlbergi* (pale morph rare), appears to be a derived character of that clade rather than an intermediate stage in the evolutionary transition from a pied (ancestral) to an all dark plumage as it is seen in *Lophaetus* and *Aquila* species.

Phylogenetic conservatism is also evident in the transition from juvenile to adult plumage. The whitish, largely unmarked juvenile underparts typical of all basal lineages among Aquilini as well as the *Harpia–Morphnus* lineage was modified only relatively recently in the *Lophaetus–Hieraaetus–Aquila* clade, where it led to a much less obvious differentiation between juvenile and adult plumages.

4.5. Biogeography

Our phylogenetic results show that the *Harpia/Morphnus* and *Spizaetus* lineages of Neotropical eagles are not each other's closest relatives. In the most parsimonious biogeographic scenario, New World *Spizaetus* are derived from Old World aquiline ancestors and possibly result from a single colonization event of the New World. Fossil *Spizaetus* remains are known from the Pleistocene of western North America (Brodkorb, 1964), indicating that the genus may have invaded the Neotropics from Asia via North America. However, this must have happened long before the Pleistocene, given that genetic distances between extant Neotropical *Spizaetus/Spizastur* and their Old World sister group indicate a split roughly 8–11 million years ago (Fig. 5). Among extant Aquilini there has been only one more, very recent, colonization of the New World involving the Golden Eagle *A. chrysaetos*, which entered North America via Beringia. The New World population has since differentiated into the subspecies *A. c. canadensis*, which is fully diagnosable from the Palearctic subspecies by mitochondrial and nuclear sequences. Golden Eagles are abundantly represented at late Pleistocene deposits of Rancho La Brea tar pits, together with remains of the extinct *Spizaetus grinnelli* and a morphnine eagle, all three of which apparently coexisted at that time (Stock, 1992).

Extant diversity of aquiline eagles, however, is clearly centered in Africa and Asia from where two lineages, a small *Hieraaetus* (*H. morphnoides*) and a large *Aquila* (*A. audax*, plus its probably sister species *A. gurneyi*), have entered Australasia during or after the Pliocene (Fig. 5). A possible third Australasian lineage is represented by the extinct Haast's Eagle of New Zealand, *Harpagornis moorei*, the largest accipitrid raptor known. In a cladistic analysis of osteological characters, Holdaway (1994) placed this genus as sister to *Aquila* based on a single apparent synapomorphy. However, since Holdaway's (1994) phylogeny did not recover monophyly of Aquilini, the *Harpagornis–Aquila* relationship also seems doubtful and molecular verification would be desirable. Using ancient DNA, this might be possible given that extensive molecular sequences have been obtained from various sub-fossil moa species of approximately the same age (Bunce et al., 2003; Huynen et al., 2003).

Acknowledgments

We thank the following colleagues and institutions for providing material for this study: A.J. Baker (Royal Ontario Museum, Toronto), Z. Bernitz (University of Pretoria, South Africa), W.S. Clark, J. Cooper (British Museum Natural History, Tring), J. Fjeldså (Zoological Museum University of Copenhagen), M. Kaiser (Tierpark Berlin), D. Mancini, B.-U. Meyburg (World Working Group Birds of Prey), P. Mundy (Department of National Park and Wildlife Management, Zimbabwe), J. Norman (Victoria Museum, Melbourne), H. Shirihai, G. Verdoorn (Endangered Wildlife Trust, South Africa), and R. Yosef (International Birding and Research Center, Eilat, Israel). B. Schmidt collected the USNM *Spizastur melanoleucos* sample. C. Huddleston and D. Liebers assisted with the data analysis. Financial support for part of the study through a grant (He1792/4-1) from Deutsche Forschungsgemeinschaft to A.J. Helbig is gratefully acknowledged.

Appendix

List of all species of the tribe Aquilini. Nomenclature follows Stresemann and Amadon (1979) except for *H. ayresii* and *H. wahlbergi*, where we follow Ferguson-Lees and Christie (2001)

Genus ***Ictinaetus*** Blyth, 1843
Ictinaetus malayensis Temminck, 1822

Genus ***Aquila*** Brisson, 1760 (*Aquila chrysaetos*)
Aquila pomarina C.L. Brehm, 1831
Aquila hastata Lesson, 1834
Aquila clanga Pallas, 1811
Aquila rapax Temminck, 1828
Aquila nipalensis Hodgson, 1833
Aquila heliaca Savigny, 1809
Aquila adalberti C.L. Brehm, 1861
Aquila gurneyi G.R. Gray, 1860
Aquila audax Latham, 1801
Aquila chrysaetos Linnaeus, 1758
Aquila verreauxii Lesson, 1830

Genus ***Hieraaetus*** Kaup, 1844 (*Hieraaetus pennatus*)
Hieraaetus fasciatus Vieillot, 1822
Hieraaetus spilogaster Bonaparte, 1850
Hieraaetus ayresii Gurney, 1862[a]
Hieraaetus pennatus Gmelin, 1788[b]
Hieraaetus morphnoides Gould, 1841
Hieraaetus kienerii Geoffroy Saint-Hilaire, 1835
Hieraaetus wahlbergi Sundevall, 1851

Genus ***Spizastur*** G.R. Gray, 1841
Spizastur melanoleucus Vieillot, 1816

Genus ***Oroaetus*** Ridgeway, 1920
Oroaetus isidori Des Murs, 1845

Appendix (*continued*)

Genus ***Polemaetus*** Heine, 1890
Polemaetus bellicosus Daudin, 1800

Genus ***Lophaetus*** Kaup, 1847
Lophaetus occipitalis Daudin, 1800

Genus ***Spizaetus*** Vieillot, 1816 (*Spizaetus ornatus*)
Spizaetus africanus Cassin, 1865
Spizaetus cirrhatus Gmelin, 1788
Spizaetus nipalensis Hodgson, 1836
Spizaetus bartelsi Stresemann, 1924
Spizaetus lanceolatus Temminck & Schlegel, 1844
Spizaetus philippensis Gould, 1863
Spizaetus alboniger Blyth, 1845
Spizaetus nanus Wallace, 1868
Spizaetus tyrannus Wied, 1820
Spizaetus ornatus Daudin, 1800

Genus ***Stephanoaetus*** W.L. Sclater, 1922
Stephanoaetus coronatus Linnaeus, 1766

Names in parentheses indicate type species of polytypic genera.

[a] The name '*Hieraaetus dubius*' (Smith 1830) formerly applied to this taxon was based on a specimen of *H. pennatus* from sub-Saharan Africa (Brooke and Vernon, 1981).

[b] The population in sub-Saharan Africa is distinctly smaller than its Palearctic counterpart and was first recognized at subspecies level by Yosef et al. (2000). These authors overlooked, however, that the valid name for this taxon is *Hieraaetus pennatus dubius* Smith, 1830, the name '*dubius*' having been wrongly applied to Ayer's Hawk Eagle (*Hieraaetus ayresii*) until recently (see Brooke and Vernon, 1981).

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