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Species or subspecies? The dilemma of taxonomic ranking of some South-East Asian hawk-eagles (genus *Spizaetus*)

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Summary

A molecular phylogeny of the *Spizaetus cirrhatus* complex is presented in this study, based on two sections of the mitochondrial genome: partial sequences of the *cytochrome b* gene and of the control region (CR). The topologies derived from the two sequences are in agreement. Within *S. cirrhatus* distances are rather low (0–1.5% in *cytochrome b*). Among the *cirrhatus* subspecies the island taxa *floris*, *vanheurni* and *andamanensis* form distinct haplogroups in the CR trees, conforming to the earlier subspecific divisions which were based on morphological characters. On the other hand, the most widespread subspecies, *limnaeetus*, does not represent a monophyletic group in the gene trees and its haplogroups do not cluster according to geographic affinities. An unambiguous resolution of relationships among haplotypes and haplogroups, respectively, was not achieved, suggesting a more recent radiation of this group of hawk-eagles in the course of the last ice ages. Concerning the outgroup taxa *Spizaetus philippensis* and *Spizaetus lanceolatus*, our data indicate a clear genetic distinction between the two subspecies *S. p. philippensis* and *S. p. pinskeri*, suggesting that they should be treated as distinct species. Yet the phylogenetic relationships of the three outgroup taxa with respect to *S. cirrhatus* are ambiguous in our trees. The taxonomic consequences of applying different species concepts (BSC, PSC) are discussed. The species concept chosen would result in different conservation strategies.

Introduction

With the advent of molecular methods in avian systematics (Sibley and Ahlquist 1990, Sibley and Monroe 1990), new interest in bird taxonomy arose in many regions of the world, and in recent years bird phylogeny has been revolutionized by a plethora of DNA sequence data. Moreover, the phylogenetic species concept (Cracraft 1983, Zink and McKitrick 1995), the general lineage concept (Helbig *et al.* 2002), and current trends in the application of the biological species concept (Haffer 1994), are now beginning to influence ornithologists´ attitudes towards taxonomy of birds. Many bird taxa formerly treated as subspecies are now being considered full species by many authorities. The aim of national and international legislation, encouraged by conservation organizations, is to prevent the extinction of bird species as well as to reduce the number of globally threatened species by conserving crucial sites and habitats for birds and other fauna and flora (Stattersfield *et al.* 1998). Nevertheless, there are many cases where the level of conservation concern depends heavily on taxonomic decisions (Collar 1997, Frankham *et al.* 2002, Newton 2003). For example,

subspecies are included only exceptionally in conservation efforts, mostly regional or local in scale. Thus, even if population numbers are small and the areas restricted, there is no legislative basis for the protection of subspecies (even if these are arguably specifically distinct).

To escape from this "self-made dilemma", some ornithologists prefer to consider distinctive taxa as species. In particular, taxa restricted to islands have been raised from subspecies to species level to intensify conservation efforts (e.g. Daugherty *et al.* 1999, Boon *et al.* 2000). Time pressure for conservation measures and limited scientific material have sometimes been responsible for such decisions, which were often made without extensive morphological investigations or DNA-based studies (Stattersfield *et al.* 1998, BirdLife International 2000, Gaston 2001). Recent examples among birds of prey are Cape Verde Kite *Milvus fasciicauda*, Juan Fernandez Hawk *Buteo exsul* and Altai Falcon *Falco altaicus* (Ferguson-Lees and Christie 2001). On the other hand, there is a general problem with species concepts and their applicability to taxa which are in the dynamic process of speciation. The application of the Biological Species Concept (BSC: Mayr 1963) or the Phylogenetic Species Concept (PSC: Cracraft 1983) would result in a considerably different number of species.

In the present work we examine these problems in a genetic investigation of the phylogenetic relationships among South-East Asian hawk-eagles of the Changeable Hawk-eagle *Spizaetus cirrhatus* complex and the closely related taxa *Spizaetus philippensis* (Philippine Hawk-eagle) and *Spizaetus lanceolatus* (Sulawesi Hawk-eagle). This group of taxa are small to medium-sized eagles, largely sedentary, with a weight up to 1.8 kg. Changeable Hawk-eagle is the most widespread of the seven *Spizaetus* hawk-eagle species in South-East Asia, with a geographical distribution from India to the Lesser Sundas, and the Philippines. It inhabits savannah woodland, cultivation with trees, secondary and open primary forests from sea level up to 2,200 m a.s.l. (Thiollay *in* del Hoyo *et al.* 1994, Ferguson-Lees and Christie 2001). Of several described subspecies, six are currently recognized (Figure 1): *S. c. cirrhatus* in the Indian subcontinent, *S. c. ceylanensis* in Sri Lanka, *S. c. limnaeetus* from the Himalayan foothills through Indomalaya into the Greater Sundas and the Philippines, *S. c. andamanensis* on the Andaman Islands, *S. c. vanheurni* on Simeulue Island north-west of Sumatra and *S. c. floris* on Sumbawa and Flores (Brown and Amadon 1968, Thiollay *in* del Hoyo *et al.* 1994, Ferguson-Lees and Christie 2001). Since some of these subspecies are endemic to small oceanic islands it is very likely that they are severely threatened by habitat destruction, especially given the large areas required by large raptors. The other two species (*S. philippensis*, *S. lanceolatus*) are restricted to small islands, the Philippines and Sulawesi, respectively. They prefer primary and secondary forests from sea level to mountainous regions (Dickinson *et al.* 1991, Nurwatha *et al.* 2000, Thiollay and Rahman 2002).

S. cirrhatus differs from all the South-East Asian *Spizaetus* species by the feathering of the tarsi which terminates abruptly at the bases of the toes (Amadon 1953). Since its distribution range overlaps with all Asian *Spizaetus* representatives (after Amadon 1982), its classification as an independent species has been generally accepted, whereas all the other South-East Asian taxa (including *S. philippensis* and *S. lanceolatus*) were lumped together in a superspecies (Thiollay *in* del Hoyo *et al.* 1994). The various subspecies of *S. cirrhatus* are well differentiated in size and shape, and to a lesser degree in plumage. However, no published data are available about

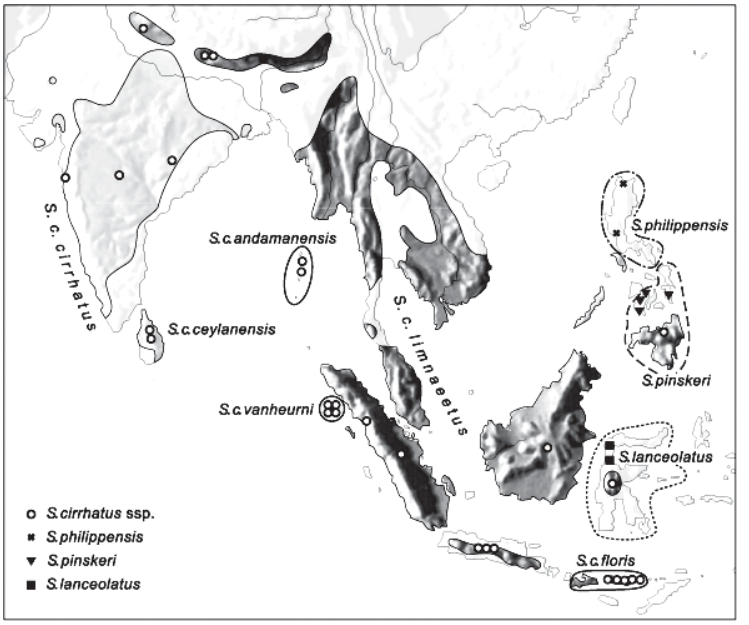

Figure 1. Distribution and localities of samples of *Spizaetus cirrhatus* (various subspecies), *S. philippensis*, *S. pinskeri* (treated as distinct species as suggested in the present study) and *S. lanceolatus*.

differentiation in vocalizations, behaviour or ecology. The largest and heaviest subspecies occur on the mainland (*cirrhatus*, *limnaeetus*) and on Sumbawa and Flores (*floris*), whereas populations of the smallest, with approximately less than half the weight (*vanheurni*), are found on the smallest island. The westernmost subspecies *cirrhatus* and *ceylanensis* are long-crested, *andamanensis* (P. Rasmussen pers. comm.) and the other subspecies are more or less un-crested. In most subspecies the ventral plumage is white with longitudinal streaks and more or less intensively barred thighs. Exceptions are the polymorphic *limnaeetus* with colours ranging from pale to melanistic, and *floris* which is almost white with a faint pattern. Because of some special features (e.g. lack of a crest, presence of a melanistic morph, vocalization) *limnaeetus* is occasionally considered to be a separate species (Amadon 1953, 1982, Stresemann and Amadon 1979, Rasmussen and Anderton 2004). The differences in plumage pattern and morphological characters are much more pronounced between the subspecies of *S. philippensis* (*philippensis*, *pinskeri*) than among the subspecies of *S. cirrhatus* (Preleuthner and Gamauf 1998, Gamauf *et al.* 1998a).

In this paper we address the following questions:

(1) Do the morphologically well differentiated *S. cirrhatus* and *S. philippensis* subspecies also differ genetically?
(2) Is *limnaeetus* a distinct species?
(3) How can the taxonomic status of the taxa investigated be interpreted using different species concepts?
(4) What would be the consequences of our data with respect to conservation?

Material and methods

Samples

Twenty-seven samples of *S. cirrhatus* with known geographical origin representing its six subspecies were examined. As outgroup taxa we used *S. philippensis* (six specimens representing two taxa) and *S. lanceolatus* (two specimens). Of the other *Spizaetus* species, these two species seem to be most closely related to *S. cirrhatus*, as will be described elsewhere (Haring *et al.* in prep.). Since it was not possible to obtain fresh tissue from most of the relevant taxa included in this study, we had to rely mainly on museum material (feathers or skin from the foot pads of study skins). Sample codes, source, collecting localities, museum inventory numbers, and GenBank accession numbers are listed in Table 1. In the case of S. c. *vanheurni* all specimens are paratypes. To assess genetic variability within taxa, up to 10 samples per taxon were analysed (e.g. *S. c. limnaeetus*). Two mitochondrial marker sequences were used which were isolated and analysed separately in two laboratories: (1) a section of the cytochrome *b* gene (*cyt b*), which was analysed at the Laboratory of the Institute for Nature Reserve (NINA, Trondheim); (2) a section of the control region (CR), which was analysed at the Laboratory of Molecular Systematics (NHM, Vienna).

DNA extraction

DNA extractions were performed following two different protocols. In one of the protocols (NHM, Vienna) a 10% Chelex (Biorad) solution containing proteinase K (0.5 mg/ml) was added. After incubation (4 h, 50°C, with agitation) solutions were heated to 95°C for 5 min and centrifuged for 1 min. For purification and to remove short fragments of degraded DNA the supernatant was purified using the QIA Quick PCR Purification Kit (Qiagen) with a final volume of 30–70 µl elution buffer. Using another extraction method (NINA, Trondheim), DNA from museum feathers was isolated according to Taberlet and Bouvet (1991) except that a Microcon YM-50 filter (Amicon) was used instead of a Centricon 30 (Amicon) to desalt and concentrate the sample. If retentate was not obtained, 10 µl of TEm: buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) was added to the Microcon YM-50 filter (Amicon), and left for 10 min before centrifugation was performed again. Control extractions with pure extraction buffer (without tissue) were prepared for the polymerase chain reaction (PCR) experiments.

PCR amplification

At the NHM, PCR was carried out with an Eppendorf Thermocycler, in a volume of 25 µl, containing 1 unit Dynazyme DNA polymerase (Finnzymes OY) 0.5 µM of each primer, and 0.2 mM of each dNTP. Initial denaturation (95°C, 2 min, was followed by 30 reaction cycles: 95°C (10 s), annealing temperature (10 s), 72°C (30 s); final extension at 72°C (5 min). At NINA PCR was performed in a 25 µl reaction mix containing 15 pmol of each primer, 2.0 mM $MgCl_2$, 0.8 mM of each dNTP, 1 µl 10× PCR buffer II and 1–1.25 U AmpliTaq Gold polymerase (Applied Biosystems) or HotStar Taq (Qiagen). After denaturation and activation of AmpliTaq Gold (10 min at 95°C) or HotStar Taq (15 min at 95°C), 40 cycles of 30 s at 94°C, 45 s at 50°C and 90 s at 72°C were performed on a 2600 or 2700 thermocycler (Applied Biosystems).

Table 1. Sample list of *Spizaetus* hawk-eagles. *S. philippensis* and *S. pinskeri* are treated as distinct species as suggested in the present study.

Taxon	Sample code	Tissue	Locality, Year	Source, Voucher	Marker	GenBank Accession nos.
Spizaetus c. cirrhatus	Scircir3	fe	India, Bombay, 1874	BMNH 1887.11.1.342	*cyt b*, CR	AY701097, AY701067
	Scircir4	pa	India, –	NMW 85.060	CR	AY701068
	Scircir5	fe	India, Seoni, 1869	BMNH 1885.8.19.1424	*cyt b*, CR	AY701098, AY701069
S. c. ceylanensis	Scircey1	pa	Sri Lanka	AMNH 534.913	*cyt b*	AY701099
	Scircey5	fe	Sri Lanka	RMNH 5210	*cyt b*, CR	AY701100, AY701070
S. c. andamanensis	Scirand1	fe	India, Andaman Is., 1874	BMNH 1885.8.19.1466	*cyt b*, CR	AY701113, AY701079
	Scirand4	fe	India, Andaman Is., 1997	B. Miller	*cyt b*, CR	AY701112, AY701080
S. c. limnaeetus	Scirlim1	pa	Indonesia, Java, 1931	NMW 322	*cyt b*, CR	AY701101, AY701071
	Scirlim2	pa	Indonesia, Borneo, Bongas/Borabei, 1882	NMW 46. 498	*cyt b*, CR	AY701103, AY701072
	Scirlim3	pa	Indonesia, Sumatra, Deli, 1886	NMW 71.242	*cyt b*, CR	AY701102, AY701073
	Scirlim4	pa	Indonesia, Java, 1884	NMW 71.241	*cyt b*, CR	AY701104, AY701074
	Scirlim9	fe	Nepal (before 1859)	BMNH 1859.3.4.603	*cyt b*	AY701105
	Scirlim10	fe	Nepal (before 1859)	BMNH 1859.3.4.602	*cyt b*, CR	AY701106, AY701075
	Scirlim11	pa	Indonesia, Java, 1924	RMNH 14000	*cyt b*, CR	AY701107, AY701076
	Scirlim12	pa	Indonesia, Sumatra, 1919	RMNH 15387	*cyt b*	AY701108
	Scirlim12a	pa	N India, Uttar Pradesh, Kalnahi, 1947	AMNH 461.932	*cyt b*, CR	AY701109, AY701077
	Scirlim13	fe	Indonesia, Sulawesi, 1935	RMNH 7208	*cyt b*, CR	AY701110, AY701078
	Scirlim16a	fe	Philippines, Mindanao, 1993	P. v. Nievenhuis	*cyt b*	AY701111
S. c. vanheurni	Scirvan1	pa	Indonesia, Simalur Is. (= Simeulue), 1913	RMNH 87.258, paratype	*cyt b*, CR	AY701114, AY701081
	Scirvan2	pa	Indonesia, Simalur Is., 1913	RMNH 87.259, paratype	*cyt b*, CR	AY701115, AY701082
	Scirvan3	pa	Indonesia, Simalur Is., 1913	RMNH 87.257, paratype	*cyt b*, CR	AY701116, AY701083
	Scirvan4	pa	Indonesia, Simalur Is., 1913	RMNH 87.260, paratype	*cyt b*, CR	AY701117, AY701084
S. c. floris	Scirflo1	pa	Indonesia, S Flores, –	AMNH 534.895	*cyt b*, CR	AY701118, AY701085

Table 1. *Continued.*

Taxon	Sample code	Tissue	Locality, Year	Source, Voucher	Marker	GenBank Accession nos.
	Scirflo2	fe	Indonesia, Flores, Waeriko, 1976	RMNH 81.112	*cyt b*, CR	AY701119, AY701086
	Scirflo3	fe	Indonesia, Flores, –	RMNH 66.257 (4)	*cyt b*, CR	AY701120, AY701087
	Scirflo4	pa	Indonesia, Flores, Sika, 1891	RMNH 16 XIV 1892	*cyt b*, CR	AY701121, AY701088
	Scirflo6	fe	Indonesia, Flores, 2002	J.-O. Gjershaug, N. Røv	*cyt b*, CR	AY701122, AY701089
S. lanceolatus	Slan1	pa	Indonesia, Sulawesi, 1931	ZMB 33.114	*cyt b*, CR	AY701129, AY701095
	Slan3	fe	Indonesia, Sulawesi, 1887	BMNH 1887.11.1.337	*cyt b*, CR	AY701130, AY701096
S. philippensis	Sphiphi2	pa	Philippines, N Luzon, 1895	BMNH 1897.5.13.311	*cyt b*, CR	AY701124, AY701090
	Sphiphi6a	fe	Philippines, Luzon, Manila, 1985	J. A. James	*cyt b*, CR	AY701123
S. pinskeri	Sphipin8	fe	Philippines, Negros, –	NMW, F-399	*cyt b*, CR	AY701125, AY701091
	Sphipin9	fe	Philippines, S Negros, –	NMW, F-400	*cyt b*, CR	AY701126, AY701092
	Sphipin10	fe	Philippines, Negros, –	NMW, F-401	*cyt b*, CR	AY701127, AY701093
	Sphipin11	fe	Philippines, ?Leyte, –	NMW, F-402	*cyt b*, CR	AY701128, AY701094

Tissue: fe, feathers; pa, skin of foot pad.

Optimal amounts of template DNA extracted from museum material were determined empirically (2–10 µl of the DNA solution) using occasionally dilutions from 1- to 50-fold (from control DNA extracted from blood samples 50 ng DNA template was used). If necessary, re-amplifications were performed with 1–2 µl template. Negative controls for PCR reactions were carried out to screen for contaminated reagents: (1) control extractions (without DNA) instead of template; (2) reaction with A.d. instead of template. Since the major part of the study was based on tissue of museum specimens, the expected maximum length of PCR fragments was < 400 bp.

The two primers that were used both for PCR amplification and direct sequencing of the *cyt b* gene were called mt-A and mt-I. Primer mt-A (L-14970) (5′-CAA CAT CTC AGC ATG ATG AAA CTT CG-3′) was modified by Wink (1998) based on the *cyt b* sequence by Kocher *et al.* (1989). Primer mt-I (H-15350) (5′-TGC TGA GAA TAG GTT GGT GAT GAC-3′) was isolated and optimized at NINA Trondheim; it was based on *cyt b* sequences of five *Aquila* species (Seibold *et al.* 1996). Of the 381 bp PCR product obtained, 264 bp corresponding to positions 15034 to 15298 of the *Gallus gallus* f. *domesticus* mitochondrial genome (Desjardins and Morais 1990) was used for sequence comparisons. For the control region two primer pairs were used: CR5+: 5′-CCC CCC CTT CCC CCC C-3′, CR7–: 5′-GAC CGA CTA AGA GAT AAC CTA-3′ (annealing temperature: 50°C), and (for specimens where no PCR product could be obtained with the primer pair CR5+/CR7–) two nested primers CR1+: 5′-ATG TAC TAT TGT ACA TTA AAC-3′, CR2–: 5′-CAA GTT ATG ACC TGC TACC-3′ (annealing temperature: 50°C).

Cloning and sequencing

PCR products were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) and cloned (TOPO TA Cloning Kit, Invitrogen). At the NHM sequencing of cloned PCR products (both directions) was performed by MWG-Biotech (Ebersberg, Germany). At NINA direct sequencing of PCR products was performed (both strands). Successful amplification and approximate quantification of PCR products were checked by running one-fifth of the PCR samples on a 2% agarose gel. PCR products were purified using Qiagen QIAquick-spin PCR purification kits (Qiagen). Approximate yields of PCR products after purification were quantified by agarose gel electrophoresis. Purified PCR products were sequenced on an Applied Biosystems 310 DNA sequencer (Foster City, CA) with *Taq* DNA polymerase and Dye Terminators or Big Dye Terminators as instructed by the manufacturer. PCR conditions for sequencing of PCR products were 30 cycles with 30 s at 96°C, 15 s at 50°C and 4 min at 60°C. The sequence extension products were purified by ethanol precipitation according to the manufacturer's instructions (Applied Biosystems) except that the ethanol was not chilled and the procedure was performed at room temperature. The sequences determined in the course of the present study are registered under the GenBank accession numbers listed in Table 1.

Sequence analysis

Alignments were produced manually. The alignments have lengths of 264 (*cyt b*) and 237 (CR) sites respectively. The reading frames of all *cyt b* sequences proved to be intact, suggesting that the sequences are derived from functional mitochondrial genes. Both distance (neighbor-joining algorithm, NJ: Saitou and Nei 1987) and maximum

parsimony (MP) methods were used to infer the phylogenetic relationships. All dendrograms were calculated with the software package PAUP (test version 4b6-10; Swofford 2002). For NJ trees uncorrected distances (p-distances) were used. MP trees were generated with heuristic search using the TBR (tree bisection reconnection) algorithm and a random taxon addition sequence (1,000 replicates). All characters were weighted equally.

Results

Since many samples consisted of only small pieces of tissue from old museum material, it was not possible in some cases to obtain sequences from both markers (Table 1). Thus, both marker sequences were sequenced from 29 samples, from five samples the *cyt b* fragment only was amplified, and from one sample the CR fragment only was obtained. The 29 samples from which both sequences could be analysed were used for the combined trees as well as for comparisons of distances and nucleotide diversity between markers.

As was expected, sequence variability of the protein-coding *cyt b* gene was lower than that of the non-coding CR sequence. For example, within the *S. cirrhatus* complex up to five substitutions (range of p-distances 0.0–1.9%; number of segregating sites: 8) were found among *cyt b* sequences (264 bp), and up to 17 substitutions (range 0.0–7.5%; number of segregating sites: 37) among the CR sections (237 bp). Nucleotide diversity per site was 4 times higher in the CR than in *cyt b* (3.4 vs 0.8). Almost no length variation was observed; only the CR sequence of the specimen Scirlim3 carried a 2 bp deletion. Between ingroup and outgroup taxa (*S. p. philippensis*, *S. p. pinskeri*, *S. lanceolatus*) the p-distance range was 2.7–6.3% for *cyt b* and 3.9–10.4% for the CR. Comparing the ranges of p-distances within and between subspecies (Table 2) it becomes apparent that within *S. c. limnaeetus* the variability is in the same range as that found between subspecies.

Among the 34 *cyt b* sequences 11 haplotypes can be distinguished, eight of them within *S. cirrhatus*. As can be seen in the NJ tree calculated from these sequences (Figure 2), two of the island taxa possess their own haplotype (*S. c. floris*), or belong to the same haplogroup (*S. c. vanheurni*). Two of the remaining five haplotypes are shared by two taxa each (*S. c. limnaeetus* + *S. c. andamanensis*, *S. c. cirrhatus* + *S. c. ceylanensis*). The *cirrhatus* complex can be divided into two groups, one comprising *S. c. cirrhatus*, *S. c. ceylanensis* and *S. c. floris*, the other comprising the other taxa (*S. c. limnaeetus*, *S. c. vanheurni*, *S. c. andamanensis*). Nevertheless, relationships between these groups as well as between haplogroups are only poorly supported in the bootstrap analyses (NJ and MP). The two shortest trees found in the MP analysis (TL = 29, CI = 0.828, RI = 0.951, RC = 0.787) have the same topology as the NJ tree (differences between the two MP trees are found only with respect to the outgroup taxa).

Among the 30 CR sequences, 24 haplotypes were found, 21 of which were among the 23 individuals of *S. cirrhatus*. The NJ tree derived from the CR sequences is depicted in Figure 3. The haplotype distribution resembles that found for *cyt b*. Again *S. c. floris* and *S. c. vanheurni* belong to separate haplogroups. In addition, the haplotypes of *S. c. andamanensis* form a distinct branch. As with *cyt b*, *S. c. cirrhatus* and *S. c. ceylanensis* form a clade. But, in contrast to the *cyt b* tree, where these two taxa cluster with *S. c.*

Table 2. Ranges of pairwise p-distances within and between taxa. *S. philippensis* (Sphi) and *S. pinskeri* (Spin) are treated as distinct species as suggested in the present study.

cyt b	Scircir	Scircey	Scirflo	Scirlim	Scirand	Scirvan	Slan	Sphi	Spin
Scircir	0.0	0.0	0.4	0.4–1.2	0.8	1.1–1.5	4.3	3.1	4.7
Scircey		0.0	0.4	0.4–1.2	0.8	1.1–1.5	4.3	3.1	4.7
Scirflo			0.0	0.8–1.9	1.1	1.5–1.9	4.7	3.5	5.1
Scirlim				0.0–1.1	0.0–0.8	0.4–1.5	3.9–5.1	2.7–3.9	5.1–6.3
Scirand					0.0	0.4–0.8	4.3	3.1	5.5
Scirvan						0.0–0.4	4.7–5.1	2.7–3.1	5.9–6.3
Slan							0.0	3.9	4.3
Sphi								0.0	3.5
Spin									0.0
CR									
Scircir	0.0–0.4	2.2–2.6	3.9–4.3	2.6–5.7	3.9–4.3	3.9–4.3	6.1–6.6	4.3–4.8	7.1–8.9
Scircey		0.0	4.4–4.8	3.9–7.5	5.3–5.7	4.4–5.3	8.0	6.7	9.0–10.4
Scirflo			0.0–1.3	2.1–5.7	2.6–3.9	2.1–3.4	6.6–8.4	4.8–5.2	7.5–9.4
Scirlim				1.3–5.7	3.0–5.2	2.1–3.0	7.1–9.4	3.9–7.6	6.6–9.4
Scirand					1.3	2.1–3.9	7.5–8.0	5.7–6.1	7.1–9.4
Scirvan						0.4–0.9	7.5–8.4	4.8–5.7	7.5–8.9
Slan							0.9	8.0	7.5–9.8
Sphi								0.0	4.3–5.7
Spin									0.0–2.1

Abbreviations according to Table 1.

floris, they appear as sister group of the remaining haplogroups of *S. cirrhatus*. The various *S. c. limnaeetus* haplotypes are scattered among the other haplogroups and do not form a monophyletic group nor do they cluster according to geographic affinities. Bootstrap support is generally low, especially for the relationships among the various haplogroups. The topologies of the NJ and MP trees are in agreement: the 155 shortest trees (TL = 87, CI = 0.655, RI = 0.820, RC = 0.537; bootstrap values are included in Figure 3) differ mainly with respect to the branching pattern of haplogroups within *S. c. cirrhatus*.

The topology of the combined tree (concatenated *cyt b* and CR sequences) is similar to the CR tree with slightly higher bootstrap support, but still the relationships among most haplogroups are poorly supported.

A surprising result of this investigation is the clear genetic distinction between *S. p. philippensis* (from Luzon) and *S. p. pinskeri* (from Negros, Leyte and Mindanao). Distances between these two taxa are within the range (*cyt b*: 3.4%, CR: 4.5–5.8%) observed between each of them and either *S. lanceolatus* or *S. cirrhatus* (*cyt b*: 2.7–5.3%, CR: 4.1–9.5%). The relationships among the three taxa used as outgroups are not unambiguously resolved. In the *cyt b* trees *S. p. philippensis* and *S. p. pinskeri* do not even form a monophyletic group.

Discussion

The molecular phylogeny of the *S. cirrhatus* complex established in this study is based on two sections of the mitochondrial genome. Despite their different levels of variability the topologies derived from the two sequences are in agreement.

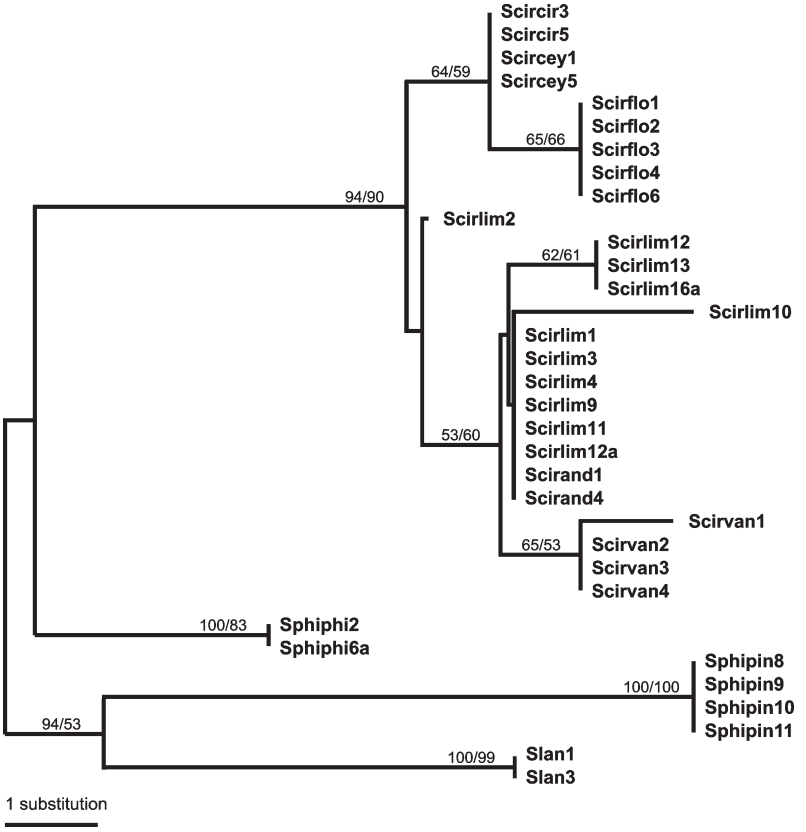

Figure 2. NJ tree based on *cyt b* sequences (midpoint rooting). Bootstrap values (1,000 replicates) >50% are given at the nodes (left: NJ, right: MP). Assignment of sequences is according to specimens in Table 1.

Genetic differentiation at species level

Although current classification places *S. lanceolatus* and *S. philippensis* either within the *S. nipalensis* "Formenkreis" (Stresemann 1924) or considers them a superspecies together with the other South-East Asian species (Amadon 1982, Thiollay *in* del Hoyo *et al*. 1994), preliminary sequence comparisons with other *Spizaetus* species (Haring *et al*. unpublished data) revealed that they are rather closely related to *S. cirrhatus*. This association is also supported by the white juvenile plumage shared by this assemblage, whereas the remaining South-East Asian hawk-eagle taxa (*S. nipalensis*, *S. alboniger*, *S. nanus*, *S. bartelsi*) have buff to orange-brown plumage at this age (Brown and Amadon 1968, Ferguson-Lees and Christie 2001). Consequently, they were taken as outgroup taxa for this study. Moreover, the two taxa can be used for comparisons of intra- and interspecific variability within this genus. As our data show, there is a clear genetic distinction between *S. p. philippensis* and *S. p. pinskeri*. Yet the phylogenetic relationships of the three outgroup taxa with respect to *S. cirrhatus* are not unambiguous in our trees, and *S. philippensis* does not even form a monophyletic group in all trees. Because of the high sequence divergence between the two lineages of *S. philippensis*, together with their clear-cut morphological and plumage pattern

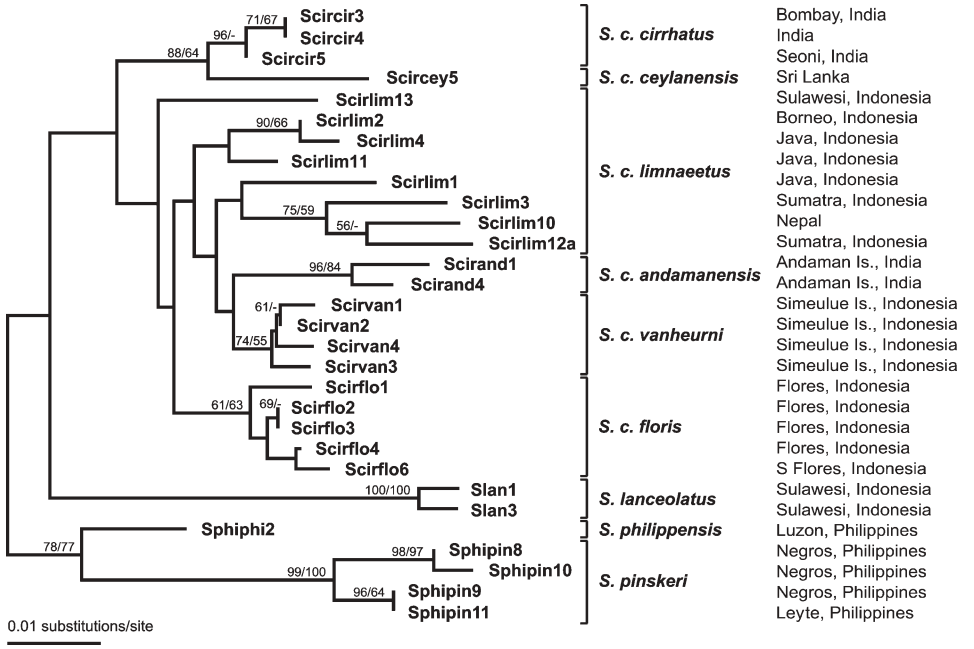

Figure 3. NJ tree based on CR sequences (midpoint rooting). Bootstrap values (1,000 replicates) >50% are given at the nodes (left: NJ, right: MP). Assignment of sequences is according to specimens in Table 1. *S. philippensis* and *S. pinskeri* are treated as distinct species as suggested in the present study.

differences (Preleuthner and Gamauf 1998), we suggest that they should be treated as distinct species.

Genetic differentiation within S. cirrhatus

Within *S. cirrhatus* the distances observed are in a range that can be expected at the intraspecific level. For example, within Eastern Honey-buzzard *Pernis ptilorhyncus* the distances for the same *cyt b* section measured 2.1–3.9% between subspecies groups and 1.5–1.8% between subspecies within groups (Gamauf and Haring 2004). In *S. cirrhatus* variability within subspecies is highest in *S. c. limnaeetus*, which has the widest geographical range including numerous islands and is also the taxon with the highest plumage variability. Lower variability is found within subspecies restricted to islands. In the *cyt b* sequences the number of parsimony-informative sites is rather low (0–5), providing only limited phylogenetic information. The relationships among *cyt b* haplogroups are not clearly resolved and bootstrap support is very low. With the exception of *floris* and *vanheurni* no clear separation of subspecies is obtained with this marker, and even these two subspecies are combined by only one synapomorphic substitution. Whether this substitution can be used as a diagnostic site has to be investigated in a larger sample of specimens. In the CR trees the island taxa *floris* and *vanheurni* (as in *cyt b*), as well as *andamanensis*, form separated haplogroups confirming the subspecific division based on morphological characters. Yet, even with

this highly variable section a clear resolution of relationships among haplotypes and haplogroups, respectively, was not achieved. The island forms appear as haplogroups within the bush-like tree of *limnaeetus* sequences, which does not represent a monophylum. There is no indication of recent gene flow between the island subspecies and *limnaeetus* or *cirrhatus*; however, the sample sizes are comparatively small. In any case, the differentiation of a western group formed by *cirrhatus* and *ceylanensis* becomes apparent, a pattern corresponding partly to that found in the *cyt b* trees. However, with the latter marker this group is associated with *floris*, although this affiliation is based on a single synapomorphic site only.

Phylogeographic considerations

The *S. c. cirrhatus-limnaeetus* complex may be another example of continuing speciation in birds in South-East Asia, apparently influenced by glacial processes. Similar scenarios have been described for the genus *Pernis* (Gamauf and Haring 2004) and several other bird groups (e.g. Dickinson *et al.* 1991). What kind of conclusions do the sequence data allow with respect to phylogeography? The close association of *S. c. cirrhatus* and *S. c. ceylanensis* (sequence identity within *cyt b*) reflects the geographic proximity of their ranges (India and Sri Lanka), assuming their rather recent separation from each other at the end of the last glaciation when the land connection was severed. The remaining taxa belong to a group that could be designated as "*limnaeetus* radiation", which can also be assumed to have occurred in the more recent past (in the course of one of the last glaciations). The division between the two groups may have occurred during glaciation of the Himalaya massif and its foothills, splitting up the ancestral population into a western (India) and an eastern (South-East Asia) range. In such cold periods with low sea level, the ancestral form of the *limnaeetus* group may have spread throughout the exposed land masses of the Sunda Shelf including the islands of Sumatra, Java and Borneo. These areas were presumably covered mainly by steppe habitats, and dispersed forest "islands" may have been located mainly on the former island areas (van Oosterzee 1997, Wilson *et al.* 2000). Such forest patches were already inhabited by the ancestors of the other South-East Asian *Spizaetus* representatives (e.g. *S. philippensis*, *S. pinskeri*, *S. lanceolatus*, *S. nanus*, *S. bartelsi*, *S. alboniger*). Supposing that the ancestor of the *limnaeetus* group was morphologically adapted to semi-open habitats as it is today (Brown and Amadon 1968, Thiollay 1988, 1996, 1998, Gamauf *et al.* 1998a, b), competition was minimized between the inhabitants of forests and the new invaders. At the end of the last glaciation this continuous geographical range may have split up again due to rising sea levels. Assuming that this species crosses only narrow sea passages, the populations became more or less isolated on their respective islands (e.g. Greater and Lesser Sundas, Andamans, Simeulue), which are at the outermost border of the distribution range. For *S. philippensis* and *S. pinskeri* it could be hypothesized that an ancestral population was subdivided earlier in the Pleistocene into two lineages, one on Greater Mindanao and the other on Greater Luzon. Factors identified as influencing species richness on the Philippines include island area, maximum elevation, and Pleistocene patterns of connection and isolation. In a similar way many other bird groups on the Philippines, especially endemic species, underwent differentiation on these two main island complexes (Peterson *et al.* 2000).

Regarding *limnaeetus*, so far there is no record of this subspecies on Sulawesi (White and Bruce 1986, Coates and Bishop 1997). The specimen Scirlim13 from Sulawesi (RMNH 7208), which had been originally designated as *S. lanceolatus*, turned out to belong to *S. c. limnaeetus* (based on comparisons of plumage characters and measurements carried out by R. Dekker, Naturalis, Leiden). Thus, it seems likely that *limnaeetus* is also distributed on that island. The misidentification had probably happened because of the white head and underparts of the juvenile plumage of this specimen, a character which both taxa share.

Species concepts and taxonomy of Changeable Hawk-eagle

Genetic diversity is recognized as the fundamental component of biodiversity (Moritz and Faith 1998). How can we use knowledge about genetic diversity and differentiation to draw conclusions concerning taxonomy or to answer the question of what a species is? A wide range of sequence divergences between pairs of presumably closely related bird species (0.1–10.6%, mitochondrial markers) and subspecies (0.1–2.6%) has been reported in Seibold and Helbig (1995). This indicates that, on the one hand, speciation may occur at different levels of sequence divergence. Moreover, species which, according to morphological similarities, appear to us as closely related may in fact have diverged a long time ago. Some examples for sequence divergences (*cyt b*) between birds of prey are 1.8% for Spanish Imperial Eagle *Aquila adalberti* versus Imperial Eagle *A. heliaca*, as well as Greater Spotted Eagle *A. clanga* versus Lesser Spotted Eagle *A. pomarina*. White-tailed Sea-eagle *Haliaeetus albicilla* and Bald Eagle *H. leucocephalus* differ by 2.5% (Seibold and Helbig 1995), and two honey buzzard species *Pernis celebensis* and *P. ptilorhyncus* by 4.0–5.7% (Gamauf and Haring 2004). Nevertheless, there is no direct way to deduce species status from observed sequence divergence values.

Although many definitions exist for the term "species" (Amadon and Short 1992, Haffer 1992, 1994), none of them are universally accepted. The Biological Species Concept (BSC: Mayr 1963) is that predominantly used in evolutionary ornithology. According to the BSC a species represents a group of interbreeding (or potentially interbreeding) natural populations that are reproductively isolated from other such groups. Since speciation can occur gradually over longer periods of time, the BSC also includes a dynamic aspect of gene pool differentiation allowing the designation of subspecies (Futuyma 1998). These are groups of populations that share a unique geographic range and/or habitat and are distinguishable from other subdivisions of the species by multiple, independent, genetically based traits (Avise and Ball 1990, O´Brien and Mayr 1991). The BSC has been increasingly challenged in recent years and a number of alternative species concepts have been proposed, which will not be treated here in detail. Island-rich South-East Asia represents a worst-case situation for the BSC, which meets its most serious challenges in insular situations (Zink and McKitrick 1995, Peterson *et al.* 2000), where reproductive isolation cannot usually be tested. Although a recent interpretation of the BSC (AOU 1998, Helbig *et al.* 2002) allows recognition of allopatric forms as species units (in common with the PSC), classification based on molecular markers without knowledge of reproductive isolation is always arbitrary, especially if no additional data, e.g. concerning karyotypes, behaviour or vocalizations, exist (Frankham *et al.* 2002). Such additional information which could serve as hints for possible reproductive isolation between the allopatric

forms of the Changeable Hawk-eagle are not yet available. At the moment, the only known barrier between the taxa is due to the isolation on islands.

In practice, two morphologically differentiated allopatric populations are often considered as distinct species if their degree of genetic differentiation matches that of two well-recognized species in a related group (e.g. *Cyanopica*: Fok *et al.* 2002). Within the *cirrhatus* complex the differences between subspecies are in the lower range observed so far for other pairs of raptor species. However, low genetic differences do not exclude species status. Between *Haliaeetus leucogaster* and *Haliaeetus sanfordi*, for example, sequence divergence is only 0.3%, although species status of these taxa may be a matter of dispute.

Nevertheless, the low genetic diversity within *S. cirrhatus* could also be interpreted as intraspecific variation for two reasons: (1) the most widespread subspecies *S. c. limnaeetus* does not represent a monophyletic group in the gene trees, and (2) its haplogroups do not cluster according to geographic affinities. Although the subspecies are geographically isolated, there is no evidence for reproductive barriers. Thus, applying the BSC there is no good argument to split this taxon into separate species (as suggested by e.g. Stresemann and Amadon 1979), although morphological differences exist (e.g. between *S. c. cirrhatus* and *S. c. limnaeetus*). At best, the sequence data suggest a separation of the western forms (*S. c. cirrhatus*, *S. c. ceylanensis*) from the rest.

Concerning *S. philippensis*, sequence data as well as clear morphological differentiation would justify the split into two species according to the PSC. As is generally the case with most island taxa, no data about reproductive barriers required by the BSC are available. However, we know from other examples that the separation between the northern and southern Philippine islands (Greater Luzon and Greater Mindanao during periods of low sea level) has led to speciation in many bird taxa (e.g. Dickinson *et al.* 1991, Peterson *et al.* 2000).

In the Phylogenetic Species Concept (PSC: Cracraft 1983) a species is considered as the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent. With this definition the PSC evades the problem of reproductive isolation between diagnosable allopatric forms by treating them all as species. Taking into account the clear morphological differences, the six taxa comprising the *S. c. cirrhatus* group would deserve species status under the PSC. Accordingly, the more or less erratic haplotype distribution would be the consequence of incomplete lineage sorting. Also the split of *S. philippensis* into two species would be in accordance with the PSC.

We have tried to show that two different species concepts (BSC, PSC) allow different conclusions. Since even among the authors there are different points of view concerning interpretation of species concepts, there is a dilemma how to come to a final decision concerning the taxonomy of this group. In this paper we advocate the maintenance of the current taxonomy of the *cirrhatus-limnaeetus* complex, which is more in accordance with the BSC. Nevertheless, in a separate paper three of the authors of the present paper (Gjershaug *et al.* 2004) will give additional information on the morphological differences and reproductive isolation between the subspecies *floris* and *limnaeetus*.

Conservation

"Correct" diagnosis of the taxonomic status of populations is critical with respect to conservation, because unrecognized species may – due to a lack of protection – become

extinct. Taking into account that species usually are given more importance in conservation measures as "evolutionary significant units" (ESU: Ryder 1986, Moritz 1994, Frankham *et al*. 2002), conservation would be more affected by further progress of the PSC than any other discipline (Collar 1997). What kind of consequences does the application of different species concepts have with respect to the conservation of the *Spizaetus* hawk-eagles? The status of the monotypic *S. lanceolatus* will not be influenced by our results, since Sulawesi still has a high proportion of rain forest (*c*. 60%; Collins *et al*. 1991), and this species still seems to be rather common (Nurwatha *et al*. 2000, Thiollay and Rahman 2002). The situation is different in the Philippines, where the splitting of Philippine Hawk-eagle into two species, *S. philippensis* and *S. pinskeri*, will lead to lower estimates of population sizes: about 200–220 pairs for *S. philippensis* on Luzon and 320–340 pairs for *S. pinskeri* on Mindanao (Preleuthner and Gamauf 1998). BirdLife International has already classified the "former" Philippine Hawk-eagle (inclusive *S. pinskeri*) as "Vulnerable" (BirdLife International 2000, Collar 2001). Meanwhile, since the mid-1990s, its rainforest habitat has continuously diminished, and it can be assumed that its population status corresponds to this decline. The populations of *S. pinskeri* endemic to Negros and a few other islands (e.g. Mindanao, Leyte, Samar) are probably also very small. In practice, the splitting of *S. philippensis* into two independent species means that, following the definition of BirdLife International (Stattersfield *et al*. 1998), both taxa should be included in the higher category "Endangered".

The most far-reaching consequences of the application of different species concepts would concern the taxa of the *cirrhatus-limnaeetus* complex with its six morphologically distinct forms. Following the current taxonomy (Ferguson-Lees and Christie 2001) based on the BSC (no evidence for reproductive barriers), *S. cirrhatus* is classified as a single species comprising six subspecies, which as a whole would not be considered threatened. Applying the PSC and similar species concepts, which differentiate all diagnosable "lineages", would lead to an upgrading of the six subspecies to species level, of which three or four would then be considered threatened. It is undisputed that they would deserve protection: three of the taxa are distributed over very small restricted ranges (Stattersfield *et al*. 1998). For example, *andamanensis* inhabits less than 6,475 km^2, *vanheurni* 1,600 km^2 and *floris* <50,000 km^2. Taking into account that birds of prey in general need large home-ranges (Newton 1979) they all appear to be scarce. The only exception is *ceylanensis*, which inhabits Sri Lanka with approximately 65,000 km^2, is still relatively common and presently not threatened (Harrison 1999). Only the nominate form *cirrhatus* and *limnaeetus* are distributed over larger ranges. Common to all taxa is that they are under more or less heavy pressure and declining (Thiollay 1996, van Balen and Nijman 1998) because of habitat loss. The population of *floris*, which is considered as rare (Butchart *et al*. 1996), is estimated to be 100 pairs maximum (Gjershaug *et al*. 2004). Population numbers of the other island-restricted *S. cirrhatus* taxa are unknown, but at least *vanheurni* is probably rare as well. In general, the existing protected areas are too small, often not very well protected and represent only minor parts of the respective distribution areas (Collins *et al*. 1991, Stattersfield *et al*. 1998). Thus, protected areas do not guarantee protection of whole populations/subspecies. Moreover, there are many taxa, currently threatened and unambiguously good species, for which "immediate protection measures" are still wanting.

Nevertheless, it would not be advisable to propose species status for such comparatively young taxa just for conservation reasons. Such a practice, which could be termed the "conservation species concept", would be a bad compromise and would have no scientific basis. High sequence similarity in one gene does not necessarily mean that these populations do not harbour genetic peculiarities which they maintain as an answer to specific environmental challenges. Morphological differences may only be the most obvious external indicators for such adaptive differentiation. Thus, even if these populations (subspecies) belonged to one biological species, they could contribute considerably to the genetic flexibility of the species and therefore should be managed as separate entities regardless of their formal taxonomic status.

The case of the *S. cirrhatus* complex may serve as an example that a change in the strategies of conservation management is required. The priority of "species" as the most important "evolutionarily significant units" should not influence taxonomists with respect to their decisions in classification. On the other hand the treatment of infraspecific taxonomic groups as evolutionarily significant units (Moritz 1994, Collar 1996) would enable the start of immediate protection measures even if taxonomic status is not clarified. When some of the various forms of Changeable Hawk-eagle have become extinct, academic discussions about the taxonomic status of its populations will no longer be relevant.

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