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Evolutionary History of New and Old World Vultures Inferred from Nucleotide Sequences of the Mitochondrial Cytochrome b Gene

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Evolutionary history of New and Old World vultures inferred from nucleotide sequences of the mitochondrial cytochrome *b* gene

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SUMMARY

The phylogeny of 11 species of Old World vultures (Aves: Accipitriformes, Aegypiinae), three species of New World vultures (Cathartidae) and their nearest relatives within and outside the order Accipitriformes was investigated based on 1026 nucleotides of the mitochondrial cytochrome *b* gene. The data support the contention that New World vultures are not birds of prey, but phylogenetic information was insufficient to identify whether they are closer to storks (Ciconiidae) or to Accipitriformes. Four species of *Gyps* are all closely related and probably speciated within the Pleistocene. Molecular data do not support the split of 'white-backed' vultures from *Gyps* in a separate genus *Pseudogyps*. The monotypic genera of large, heavy-billed vultures, *Aegypius*, *Torgos*, *Trigonoceps* and *Sarcogyps*, are of monophyletic origin. We propose to merge *Torgos* with *Aegypius*, but retain *Trigonoceps* and *Sarcogyps* as separate genera, *Sarcogyps* being clearly the most primitive of the four. All four, together with *Gyps* and *Necrosyrtes*, form a monophyletic subfamily or 'core group', to which the subfamily Aegypiinae should be restricted. This group shares a more recent common ancestor with several non-vulture genera of Accipitrids, among them *Buteo*, *Aquila*, *Haliaeetus* and *Circaetus*, than it does with the two aberrent vultures *Gypaetus barbatus* and *Neophron percnopterus*. The last two are much more primitive; they seem to be each other's sister species and are closer to *Pernis* than to other Accipitrids. We propose separating *Gypaetus* and *Neophron* in the subfamily Gypaetinae. If the cytochrome *b* gene tree accurately reflects vulture phylogeny, Old World vultures are polyphyletic with the *Aegypius–Gyps* clade having evolved convergently to the more ancient *Gypaetus* and *Neophron* vultures. Polyphyly of Old World vultures, although in conflict with the DNA–DNA hybridization phylogeny of Sibley & Ahlquist (1990), is well supported by molecular, karyotypic, morphological and other phenotypic evidence (behaviour, voice) indicating fundamental differences between the two evolutionary lines.

1. INTRODUCTION

The 'vulture' way of life, i.e. scavenging largely on dead animals, was originally thought to have evolved only once among extant diurnal birds of prey (cf. historical review of classification by Sibley & Ahlquist 1990, pp. 473–484). As early as 1873, however, it was suspected that the New World vultures Cathartidae are not true vultures (Garrod 1873). Cathartids differ most conspicuously from Accipitriform raptors in their lack of a grasping foot and lack of a syrinx, but also in many other important characteristics of skeleton, musculature, feather tracts, moult, internal organs (larynx, liver), physiology, sexual dimorphism and egg shell structure (Ligon 1967; Rea 1983). Behavioural differences between Cathartids and Accipitrid raptors are equally conspicuous, while Cathartids share apparently derived behavioural characters with storks such as defecation on the legs (for thermoregulation), bill clapping, neck sac inflation, wing spread display, interlocking of bills during copulation and incubation by both sexes (König 1982; Rea 1983). Although still placed in the Accipitriformes (or Falconiformes including Accipitridae) in most classifications up to the 1980s (see, for example: Wetmore 1960; Brown & Amadon 1968; Stresemann & Amadon 1979; Cracraft 1981), it is now well established that phylogenetically Cathartidae are not birds of prey but are more closely related to storks (Ciconiidae) (König 1982; Sibley & Ahlquist 1990). Old and New vultures, therefore, represent an impressive example of convergent evolution driven by their similar scavenging ways of life.

The Old World vultures (subfamily Aegypiinae, *sensu* Peters 1931) are typical Accipitrid raptors, but are morphologically diverse. The most recent systematic treatment (Mundy *et al.* 1992) identified a putatively monophyletic 'core group' consisting of two sister groups, one comprising *Gyps/Pseudogyps*, the other *Aegypius*, *Torgos*, *Trigonoceps* and *Sarcogyps* (figure 1). The hooded vulture *Necrosyrtes monachus* was included within this core group, but its affinities to either of the two clades remained unclear. Morphologically it resembles the Egyptian vulture *Neophron percnopterus* in possessing a long, narrow and pointed beak, suggesting

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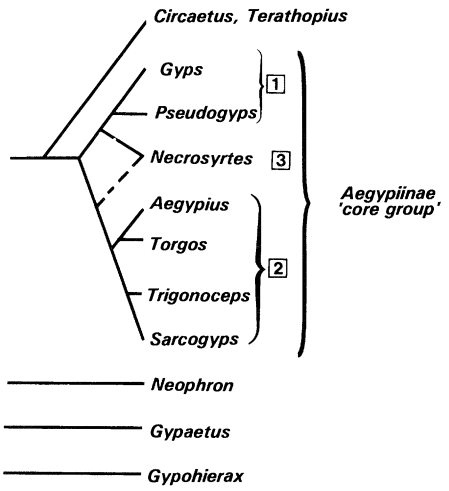

Figure 1. Hypothetical relationships of Old World vultures based on morphological characters (after Mundy *et al.* 1992).

perhaps an intermediate position between the Aegypiine core group and *Neophron*. Lammergeier *Gypaetus barbatus* and Egyptian vulture were considered not to be closely related to the Aegypiine core group (Jollie 1976/77; Mundy *et al.* 1992), thus implying a polyphyletic origin of Old World vultures. While Jollie considered *Neophron* to be 'kite-like', implying relationships to *Elanus* and *Pernis*, Thaler *et al.* (1986) found *Gypaetus* and *Neophron* to be similar to each other in several respects and probably related to *Buteo*, *Circus* and booted eagles. Mundy *et al.* (1992) tentatively unite *Neophron* and *Gypaetus* in a separate subfamily Gypaetinae.

Few other hypotheses have been proposed about the relationships of Old World vultures to other Accipitrid genera. Brown & Amadon (1968) suggested a monophyletic vulture assemblage with sea eagles (*Haliaeetus*) as their sister group. *Gypohierax* was thought to represent a transition between sea eagles on the one hand and *Neophron* and *Gypaetus*, the putatively most primitive vultures, on the other hand.

In this paper we primarily investigate the phylogeny of Old World vultures and their systematic relationships with the help of nucleotide sequences of the mitochondrial cytochrome *b* gene. We specifically test the hypothesis that Old World vultures are polyphyletic by including a number of other Accipitriform genera that might cluster as sister taxa of some but not all vultures. We were also interested to see whether our relatively short mitochondrial DNA sequences might support the exclusion of Cathartidae from Accipitriformes and perhaps corroborate their Ciconiine affinities suggested by DNA–DNA hybridization (Sibley & Ahlquist 1990) and phenotypic evidence.

2. MATERIAL AND METHODS

We used the polymerase chain reaction (PCR, Saiki *et al.* 1988) to amplify a large fraction of the mitochondrial cytochrome *b* gene (total length 1143 b.p.). A total of 1026 nucleotides was sequenced directly in three New World vultures (*Vultur*, *Sarcoramphus*, *Cathartes*), 11 species of Old World vultures of all extant genera except *Gypohierax*, representatives of eight non-vulture genera of Accipitridae, two of Falconidae (*Falco*, *Caracara*) and two species of true storks (*Ciconia*, *Leptoptilos*; table 1). Groove-billed ani (*Crotophaga sulcirostris*; Avise *et al.* 1994*a*) and common gull (*Larus canus*; K. Blechschmidt, personal communication) were included as outgroups.

Cytochrome *b* sequences of several Ciconiiform birds including New and Old World vultures published by Avise *et al.* (1994*b*) were obtained from the GenBank data base. We have independently sequenced five of the same taxa, thus allowing direct comparison of sequences. Differences between the GenBank sequences and ours were so large (*Gypaetus barbatus* 5.7%, *Leptoptilos crumeniferus* 2.9%, *Neophron percnopterus* 8.4%, *Torgos tracheliotus* 7.4%, only *Vultur gryphus* was identical) that they cannot be regarded as real. For each of the three species where divergence is largest we obtained fully concordant sequences of two to seven individuals. We therefore regard our data as well corroborated and do not include that of Avise *et al.* in our analysis.

(*a*) *DNA isolation, primer design, PCR and sequencing*

Total DNA was isolated from small blood samples obtained mostly from captive animals (zoos, bird parks) and preserved in NaF–EDTA buffer (Arctander 1988). About 100 µl of the blood–buffer mixture was incubated overnight in a lysis buffer containing 1% (by volume) SDS and 2 mg of proteinase K. Protein and cell debris were precipitated by adding one-third volume of saturated NaCl solution, after which DNA was precipitated with isopropanol, washed once with 70% (by volume) ethanol and redissolved for 1 h at 65 °C in Tris–EDTA buffer. Polymerase chain reactions (PCR) were done in a total volume of 100 µl with use of 1 µg of DNA, 50 pmol of each primer, 1.5 mM $MgCl_2$ and 2 units of Taq polymerase (Promega). After initial denaturation (2.5 min at 94 °C), 32 cycles of 30 s at 93 °C, 30 s at 45 °C and 2 min at 72 °C were run on a Biometra thermocycler.

Primer sequences (table 2) were modified and extended from those given by Kocher *et al.* (1989) to match raptor sequences as closely as possible (Seibold 1994). PCR primers A and F amplified a 1100 b.p. portion of the mitochondrial genome including most of the cytochrome *b* gene (minus 100 nucleotides at its 3′ end). Primers B–H (except F) were used as sequencing primers.

PCR products were gel-purified, redissolved in 6.5 µl of water and sequenced directly (no reamplification). Double-strand sequencing with the chain-termination method (Sanger *et al.* 1977) was done at room temperature with use of ^{35}S-dATP as the radioactive labelling nucleotide and Sequenase 2.0 (USB) according to the distributor's specifications. Sequencing reaction products were separated on a denaturing 60 g l^{-1} polyacrylamide–7 M urea gel at 65 W. The gel was dried and exposed to X-ray film for 3–4 days. Between 300 and 350 nucleotide positions were readable per sequencing reaction. Sequencing primers were spaced such that large overlaps in readable sequence of both L- and H-strand resulted, giving us an opportunity to verify sequences obtained from independent reactions.

(*b*) *Tree construction*

To estimate the phylogenetic information content of the data matrix, the skewness of the tree length distribution was assessed for a random sample of 10000 trees ('random trees' option in PAUP) by using the *g*-test (Hillis & Huelsenbeck 1992). Significant amounts of phylogenetic signal were

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Table 1. *Species of which cytochrome b gene was sequenced in this study and broad geographic origin (where known) of the birds*

(Most birds were from zoos or bird parks and the country of origin was not always known. The two columns on the right show for each species the number of individuals from which either 300 or 1026 base pairs (b.p.) of cytochrome *b* sequence data were obtained (cf. appendix).)

species	origin	300 b.p.	1026 b.p.
Accipitridae			
Buteo buteo (*buteo*, *vulpinus*) common buzzard	Germany (2), Israel (2)	2	2
Accipiter gentilis northern goshawk	Germany	1	1
Circus cyaneus northern harrier	Switzerland, Spain (2), Scotland (2)	4	1
Milvus milvus red kite	Germany (5), Switzerland (1)	5	1
Haliaeetus albicilla white-tailed eagle	northern Europe (2), Kamtschatka	1	2
Aquila chrysaetos golden eagle	Switzerland (4), not known (1)	2	3
Circaetus gallicus pectoralis short-toed eagle	S. Africa	1	1
Pernis apivorus honey buzzard	Germany (2), Switzerland, Austria	3	1
Aegypius monachus Eurasian black vulture	not known	2	1
Torgos tracheliotus lappet-faced vulture	Cape Prov., S. Africa	—	2
Trigonoceps occipitalis white-headed vulture	Africa	—	1
Sarcogyps calvus Asian king vulture	India	—	1
Necrosyrtes monachus hooded vulture	Africa	—	1
Gyps fulvus griffon vulture	Saudi Arabia (1), not known (2)	2	1
Gyps coprotheres Cape vulture	Cape Prov., S. Africa	1	1
Gyps africanus African white-backed vulture	S. Africa (1), not known (1)	1	1
Gyps bengalensis Asian white-backed vulture	India (2), not known (1)	1	2
Neophron percnopterus Egyptian vulture	India	1	2
Gypaetus barbatus lammergeier	Pyrenees, Crete, Greece, Iran (1 each) Mongolia (2), not known (1)	5	2
Falconidae			
Falco peregrinus peregrine falcon (3 subspec.)	Saudi Arabia (3), Scotland, Italy	3	2
Caracara plancus crested caracara	not known	—	2
Cathartidae			
Cathartes aura turkey vulture	S. America	—	1
Vultur gryphus Andean condor	S. America	1	1
Sarcoramphus papa king vulture	Panama	1	1
Ciconiidae			
Ciconia ciconia white stork	Germany	2	1
Leptoptilos crumeniferus marabou	Africa	—	1

Table 2. *Primer sequences (5′–3′) used for* PCR *and direct sequencing (modified from Kocher et al. 1989)*

(Positions in the chicken mitochondrial genome (Desjardins & Morais 1990) corresponding to the 3′-end of each primer are given in parentheses (L, light strand; H, heavy strand).)

PCR primers
mt-A (L-14995): CTCCCAGCCC CATCCAACAT CTCAGCATGA TGAAACTTCG
mt-F (H-16065): CTAAGAAGGG TGGAGTCTTC AGTTTTTGGT TTACAAGAC

sequencing primers
mt-B (H-15298): TTGTGATTAC TGTAGCACCT CAAAATGATA TTTGTCCTCA
mt-C (L-15320): TAYGTCCTAC CATGAGGACA AATATCATTC TGAGG
mt-D (L-15578): AAAATCCCAT TCCACCCCTA CTACTCCACA AAAGA
mt-G (L-15180): CWTCCTTMTT CTTCATCTGC ATCTAC
mt-H (L-15722): CCYCCACACA TCAAACCMGA ATGATACTTC CTATT

present in all data sets used here; so reconstruction of phylogenetic trees from these data was justified.

There is no general agreement on which method of constructing phylogenetic trees from nucleotide sequences is best (Nei 1991). We therefore compared results of two well established methods: maximum parsimony (PAUP 3.1.1; Swofford 1993) and the neighbour-joining method (Saitou & Nei 1987) as implemented in the program MEGA (Kumar *et al.* 1993). Genetic distances for the neighbour-joining analyses were estimated according to Kimura's (1980) two-parameter

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Table 3. *Genetic distances between 26 taxa studied based on 1026 nucleotides of the cytochrome b gene*

(Above diagonal, percentage sequence divergence (*p*-distance); below diagonal, Kimura two-parameter distance(%) with expected TS:TV ratio = 10:1.)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1 *Buteo buteo*	—	9.9	11.6	12.7	11.4	11.5	8.8	11.1	13.7	14.4	12.7	12.4	12.4	13.4	12.9	12.1	12.1	12.0	11.9	17.3	15.7	13.4	14.1	15.8	15.8	14.9
2 *Accipiter gentilis*	11.1	—	11.2	12.8	11.8	13.0	11.2	12.9	14.3	14.5	13.1	13.4	13.6	14.1	14.6	13.4	12.9	12.8	13.0	17.7	15.5	14.8	15.9	17.0	17.1	15.8
3 *Circus cyaneus*	13.4	12.8	—	12.5	11.8	12.0	11.4	13.1	13.3	14.0	13.3	11.7	14.2	12.6	13.2	12.3	12.2	12.3	12.0	16.4	15.2	15.0	15.7	15.9	17.0	15.3
4 *Aquila chrysaetos*	14.8	15.0	14.7	—	11.4	11.4	11.9	13.5	13.6	14.6	12.3	12.2	12.7	12.4	12.4	12.4	12.3	12.3	12.1	17.0	15.6	14.5	15.0	16.3	15.0	14.8
5 *Circaetus gallicus*	12.9	13.6	13.6	13.0	—	12.1	11.5	12.8	12.8	14.0	11.4	11.2	12.8	11.5	12.1	9.8	9.5	9.6	9.3	16.8	15.7	14.3	15.0	15.8	15.3	14.5
6 *Pernis apivorus*	13.4	15.5	14.1	13.3	14.0	—	12.1	12.1	11.6	13.2	11.5	11.2	13.1	11.9	12.8	11.5	11.4	11.3	11.2	15.2	14.4	12.5	13.4	13.8	13.8	12.8
7 *Milvus milvus*	9.7	12.7	13.0	13.7	13.1	14.1	—	10.4	12.7	13.7	13.0	11.4	12.6	12.6	12.6	12.2	12.1	12.2	12.2	15.8	15.6	14.9	15.0	15.8	15.9	15.2
8 *Haliaeetus albicilla*	12.5	15.1	15.3	15.8	14.8	14.2	11.5	—	15.4	15.8	13.5	13.3	14.3	13.5	13.7	13.2	12.9	12.6	13.0	17.3	17.8	15.0	16.2	16.0	16.3	16.1
9 *Gypaetus barbatus*	16.3	17.2	15.9	16.3	14.9	13.3	14.9	18.7	—	11.4	14.0	12.8	14.0	13.2	13.9	13.9	14.1	14.0	13.9	16.0	15.6	14.1	14.2	15.4	15.3	13.4
10 *Neophron percnopterus*	17.5	17.8	17.0	17.7	16.7	15.6	16.5	19.5	13.2	—	15.5	14.4	15.8	15.5	15.7	13.8	13.7	13.6	13.9	16.2	15.9	14.4	15.8	16.1	15.3	15.1
11 *Necrosyrtes monachus*	14.8	15.4	15.8	14.3	13.0	13.4	15.2	15.8	16.7	19.0	—	8.6	9.1	8.7	8.9	7.6	7.9	7.7	7.5	17.1	16.4	14.8	15.7	15.7	15.9	15.2
12 *Aegypius monachus*	14.4	15.8	13.6	14.2	12.7	12.9	13.1	15.6	15.0	17.4	9.4	—	8.0	4.2	5.9	7.4	8.4	7.9	7.9	16.0	15.7	13.7	15.1	15.1	14.3	14.3
13 *Sarcogyps calvus*	14.4	16.1	17.0	14.8	14.8	15.5	14.6	17.0	16.7	19.3	10.0	8.6	—	9.0	8.6	8.5	9.0	8.5	9.0	17.1	16.1	14.6	15.5	16.0	15.1	16.4
14 *Torgos tracheliotus*	15.7	16.8	14.9	14.4	13.0	13.9	14.6	16.0	15.6	18.9	9.5	4.4	9.9	—	6.4	8.4	9.5	9.2	8.8	15.7	16.3	14.8	15.5	15.9	15.5	14.9
15 *Trigonoceps occipitalis*	15.0	17.5	15.6	14.4	13.9	15.0	14.6	16.2	16.5	19.2	9.8	6.2	9.3	6.8	—	8.3	8.7	8.5	7.9	16.2	15.7	15.3	15.5	16.3	15.9	15.7
16 *Gyps bengalensis*	14.0	15.7	14.3	14.4	11.0	13.2	14.0	15.4	16.4	16.6	8.2	7.9	9.2	9.1	9.0	—	2.4	2.4	1.9	17.4	16.1	14.0	14.7	15.7	15.6	15.1
17 *Gyps coprotheres*	14.0	15.0	14.2	14.3	10.6	13.1	14.0	15.0	16.7	16.4	8.6	9.1	9.8	10.4	9.4	2.4	—	2.3	1.0	17.4	16.0	13.9	15.0	16.2	15.8	15.5
18 *Gyps africanus*	13.9	14.9	14.4	14.4	10.6	13.0	14.0	14.7	16.6	16.2	8.4	8.6	9.2	10.1	9.2	2.4	2.3	—	2.0	17.4	15.7	13.8	15.1	15.2	15.0	15.3
19 *Gyps fulvus*	13.8	15.2	13.9	14.1	10.3	12.9	14.0	15.2	16.4	16.6	8.1	8.5	9.8	9.6	8.5	1.9	1.0	2.0	—	17.0	15.9	13.9	14.6	15.6	15.3	15.2
20 *Falco peregrinus*	22.4	23.4	20.9	21.8	21.3	19.1	20.1	22.4	20.3	20.5	22.0	20.3	22.0	19.8	20.6	22.5	22.4	22.3	21.7	—	13.8	15.2	14.7	16.6	17.7	15.7
21 *Caracara plancus*	19.8	19.6	19.3	19.6	19.6	17.7	19.7	23.2	19.5	20.0	21.0	19.9	20.5	20.7	19.8	20.5	20.3	19.8	20.0	16.6	—	14.9	15.5	15.6	16.5	15.0
22 *Vultur gryphus*	16.2	18.3	18.6	17.7	17.2	14.9	18.3	18.5	17.1	17.7	18.1	16.6	17.9	18.1	18.8	17.0	16.8	16.6	16.7	18.9	18.4	—	7.4	9.3	14.6	13.2
23 *Sarcoramphus papa*	17.2	19.8	19.8	18.4	18.3	16.1	18.5	20.2	17.4	19.7	19.5	18.7	19.3	19.3	19.2	17.9	18.3	18.5	17.8	18.3	19.3	7.9	—	11.0	14.5	13.6
24 *Cathartes aura*	19.7	21.6	20.1	20.5	19.5	16.7	19.7	20.0	19.1	20.3	19.6	18.6	20.1	19.9	20.5	19.5	20.2	18.8	19.4	21.1	19.5	10.2	12.3	—	15.5	14.2
25 *Ciconia ciconia*	19.7	21.7	21.8	18.5	18.8	16.7	20.0	20.6	19.0	18.9	19.7	17.4	18.7	19.2	19.7	19.2	19.5	18.5	18.9	23.1	20.9	17.7	17.4	19.1	—	9.2
26 *Leptoptilos crumeniferus*	18.3	19.9	19.3	18.2	17.6	15.3	18.9	20.4	16.3	18.8	18.8	17.5	20.7	18.4	19.6	18.6	19.2	19.0	18.8	19.8	18.5	15.8	16.2	17.1	10.2	—

model. With PAUP, heuristic and 'branch and bound' searches were run, each with random sequence addition and including all characters. Protein coding sequences of animal mtDNA show a rapid saturation of transitional substitutions (among purines and among pyrimidines) at third and some first codon positions owing to multiple substitutions over time (Arctander 1991; Edwards *et al.* 1991; Haeseler *et al.* 1993). To eliminate this 'noise' from the sequence data, transitions were down-weighted by a factor of 0.2 (TV:TS = 5:1) or 0.05, (TV:TS = 20:1) relative to transversions (changes between a purine and a pyrimidine) in parsimony searches aimed at resolving phylogenetically old branches. The robustness of each clade within the phylogeny was assessed by running 100 bootstrap replicates (Felsenstein 1985) both with maximum parsimony (PAUP, heuristic search, random sequence addition) and neighbour-joining (MEGA, Kimura distance; for calculation of bootstrap confidence limits in MEGA see Kumar *et al.* 1993). We interpret bootstrap frequencies as heuristic levels of support for the monophyly of each clade, not as statistical significance levels.

The 26 cytochrome *b* sequences reported in this paper are available at the EMBL databank under accession numbers X86738–X86763.

3. RESULTS

(*a*) *Variation of cytochrome b sequences*

The entire sequence data set of 1026 nucleotides from 26 species (see table 1) aligned without any indels and contained no stop codons (appendix). Pairwise genetic distances between all taxa were calculated as *p*-distances (proportion of sites with nucleotide difference) with use of Kimura's (1980) two-parameter model with an expected TS/TV ratio of 10.0 (conservative estimate, see figure 2) to correct for multiple substitutions (table 3).

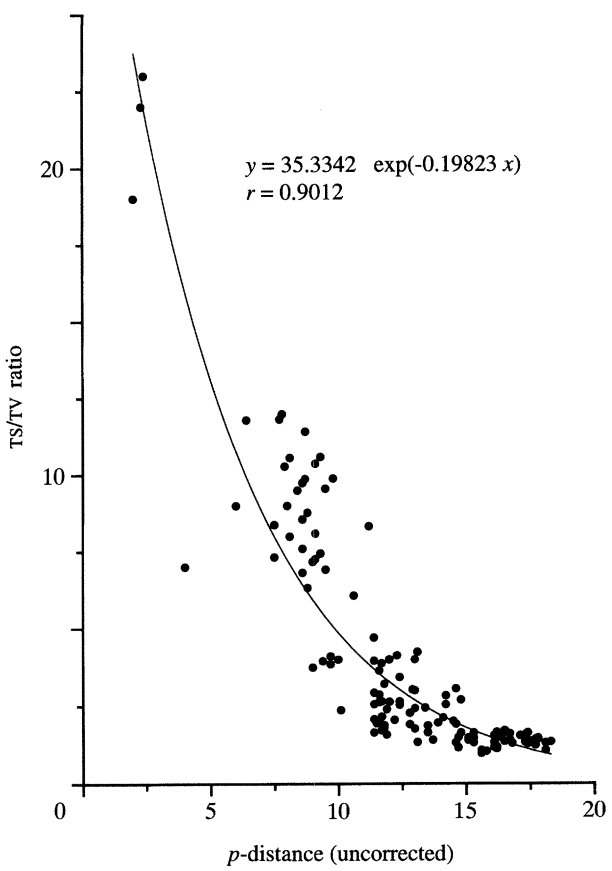

Figure 2. Relation between transition/transversion ratio and *p*-distance (proportion of nucleotides differing) for pairwise comparisons of cytochrome *b* sequences given in the appendix. With increasing genetic distance the ratio decreases exponentially owing to multiple substitutions at the same sites.

Of the 1026 nucleotide positions, 440 were variable and 358 were parsimony informative. Among the 342 amino acid sites, replacement substitutions were inferred for 69, but only 45 replacements were parsimony informative. Since this number is less than twice the number of taxa, we did not expect the amino acid sequences to be phylogenetically very informative and therefore restricted our analysis to the nucleotide data set. With respect to the frequency of observed nucleotide differences, two kinds of biases must be taken into account in phylogenetic analyses, as follows.

(*i*) *Transition/transversion (TS/TV) bias*

Among closely related taxa, transitions occurred much more frequently than transversions in our cytochrome *b* sequence data set. For instance, between closely related *Gyps* species (*p*-distance 1.0–2.4%) the TS/TV ratio ranged from 18 to 23. The ratio declined exponentially with increasing *p*-distance (figure 2) and approached values around 1 at $p > 15\%$. This relation is well known for mitochondrial sequences (Arctander 1991; Haeseler *et al.* 1993) and means that transitions are highly saturated by multiple substitutions among species that have diverged by more than 15%.

(*ii*) *Biases in codon use*

Each amino acid is coded for by up to six different codons. If selection or mutation does not favour the use of specific codons, the relative frequency of 'synonymous' codons (coding for same amino acid) should be equal. In reality, however, a taxon-specific bias in codon use is well known for many organisms (Sharp *et al.* 1988). Among the species investigated in this study, a distinct bias in codon use was found for most amino acids (table 4). It was largest in serine and leucine, each coded by six different codons. In each case, one codon (UCC for serine, CUA for leucine) was about three times as frequent as expected (table 4). For each of the 20 amino acids the two most frequently used codons differed by a C–A transversion at the third position. In keeping with this bias, C–A was by far the commonest transversional difference observed in the data set (table 5). The relative frequency bias of synonymous codons ending on A or G was more pronounced than for those ending on C or T. This should lead to a higher frequency of T–C as opposed to A–G transitions, which is exactly what we observe (table 5).

(*b*) *Overall relationships of storks, New World vultures and diurnal birds of prey*

The careful choice of outgroups is of great importance in phylogenetic analyses of nucleotide sequences. Several outgroups of decreasing phylo-

Table 4. *Cumulative codon use in all taxa sequenced in this study (see table 1)*

(Average codon frequencies and relative synonymous codon use (RSCU, in parentheses). Total number of codons, 339. Asterisk indicates a stop codon.)

UUU (F)	5.1 (0.39)	UAU (Y)	2.5 (0.37)
UUC (F)	21.3 (1.61)	UAC (Y)	10.9 (1.63)
UUA (L)	4.2 (0.40)	UAA (*)	0.0 (0.00)
UUG (L)	0.6 (0.06)	UAG (*)	0.0 (0.00)
UCU (S)	1.4 (0.41)	UGU (C)	1.2 (0.57)
UCC (S)	9.9 (2.94)	UGC (C)	2.9 (1.43)
UCA (S)	7.0 (2.08)	UGA (W)	8.2 (1.83)
UCG (S)	0.4 (0.13)	UGG (W)	0.8 (0.17)
CUU (L)	5.5 (0.53)	CAU (H)	3.1 (0.57)
CUC (L)	16.3 (1.56)	CAC (H)	7.8 (1.43)
CUA (L)	33.0 (3.15)	CAA (Q)	6.3 (1.57)
CUG (L)	3.1 (0.30)	CAG (Q)	1.7 (0.43)
CCU (P)	1.8 (0.34)	CGU (R)	0.4 (0.22)
CCC (P)	7.9 (1.52)	CGC (R)	2.9 (1.66)
CCA (P)	10.8 (2.08)	CGA (R)	3.3 (1.88)
CCG (P)	0.3 (0.07)	CGG (R)	0.4 (0.24)
AUU (I)	5.8 (0.42)	AAU (N)	2.3 (0.31)
AUC (I)	21.7 (1.58)	AAC (N)	12.2 (1.69)
AUA (M)	4.8 (1.72)	AAA (K)	6.7 (1.69)
AUG (M)	0.8 (0.28)	AAG (K)	1.2 (0.31)
ACU (T)	3.3 (0.50)	AGU (S)	0.1 (0.03)
ACC (T)	14.1 (2.08)	AGC (S)	1.3 (0.40)
ACA (T)	9.4 (1.39)	AGA (*)	0.0 (0.00)
ACG (T)	0.2 (0.03)	AGG (*)	0.0 (0.00)
GUU (V)	1.2 (0.37)	GAU (D)	1.1 (0.42)
GUC (V)	3.9 (1.18)	GAC (D)	4.0 (1.58)
GUA (V)	7.5 (2.27)	GAA (E)	5.6 (1.60)
GUG (V)	0.6 (0.17)	GAG (E)	1.4 (0.40)
GCU (A)	3.4 (0.57)	GGU (G)	2.0 (0.33)
GCC (A)	13.3 (2.21)	GGC (G)	9.8 (1.60)
GCA (A)	6.9 (1.15)	GGA (G)	9.9 (1.62)
GCG (A)	0.5 (0.08)	GGG (G)	2.7 (0.44)

Table 5. *Relative frequency (%) of the six possible nucleotide differences*

(Values were determined by all pairwise comparisons among cyt *b* sequences of 26 taxa listed in table 1 ($n = 44028$ differences.)

	G	C	T
A	18.7	23.0	7.0
G	—	2.8	1.0
C		—	47.4

genetic distance from the ingroup arranged as 'Hennig's comb' are thought to be optimal. To establish polarity (from primitive to derived groups) reliably within the total data set, we first studied the overall relationships of storks, New World vultures, falcons and Accipitrids. In a first analysis with *Gallus gallus* (Desjardins & Morais 1990) as outgroup it was established that the ani (*Crotophaga*) and the gull (*Larus*) are indeed successively more closely related to the ingroup (tree not shown), as was expected from the DNA–DNA hybridization phylogeny (Sibley & Ahlquist 1990). Thus *Crotophaga* and *Larus* are valid outgroups for studying the phylogeny of storks and birds of prey, while the chicken was dropped from further analyses, because it is too distant to provide much polarity information for the ingroup.

Parsimony analyses were run with transversions weighted 5:1 over transitions and with various combinations of these outgroups. With respect to the relationships of storks, Cathartids, falcons and Accipitrids, the following results were obtained (figures 3 and 4).

(1) Accipitridae (including *Buteo*, *Accipiter*, *Circus*, *Haliaeetus*, *Aquila*, *Circaetus*, *Pernis* and eight genera of Old World vultures) formed a monophyletic group to the exclusion of Falconidae, Cathartidae and storks (bootstrap frequency 97–99%).

(2) Falconidae and Accipitridae clustered as sister groups to the exclusion of Cathartidae and storks, although with poor bootstrap support (59–69%).

(3) It was not possible to determine with any degree of certainty which of the taxa included is the sister group of diurnal birds of prey (Accipitridae and Falconidae). This position was usually occupied by the Cathartidae, but bootstrap support was always below 60%.

(4) The three species of Cathartids formed a well supported monophylum distinct from both storks and Accipitriform raptors (bootstrap frequency 100%). The genetic distance data (table 3) show that the three

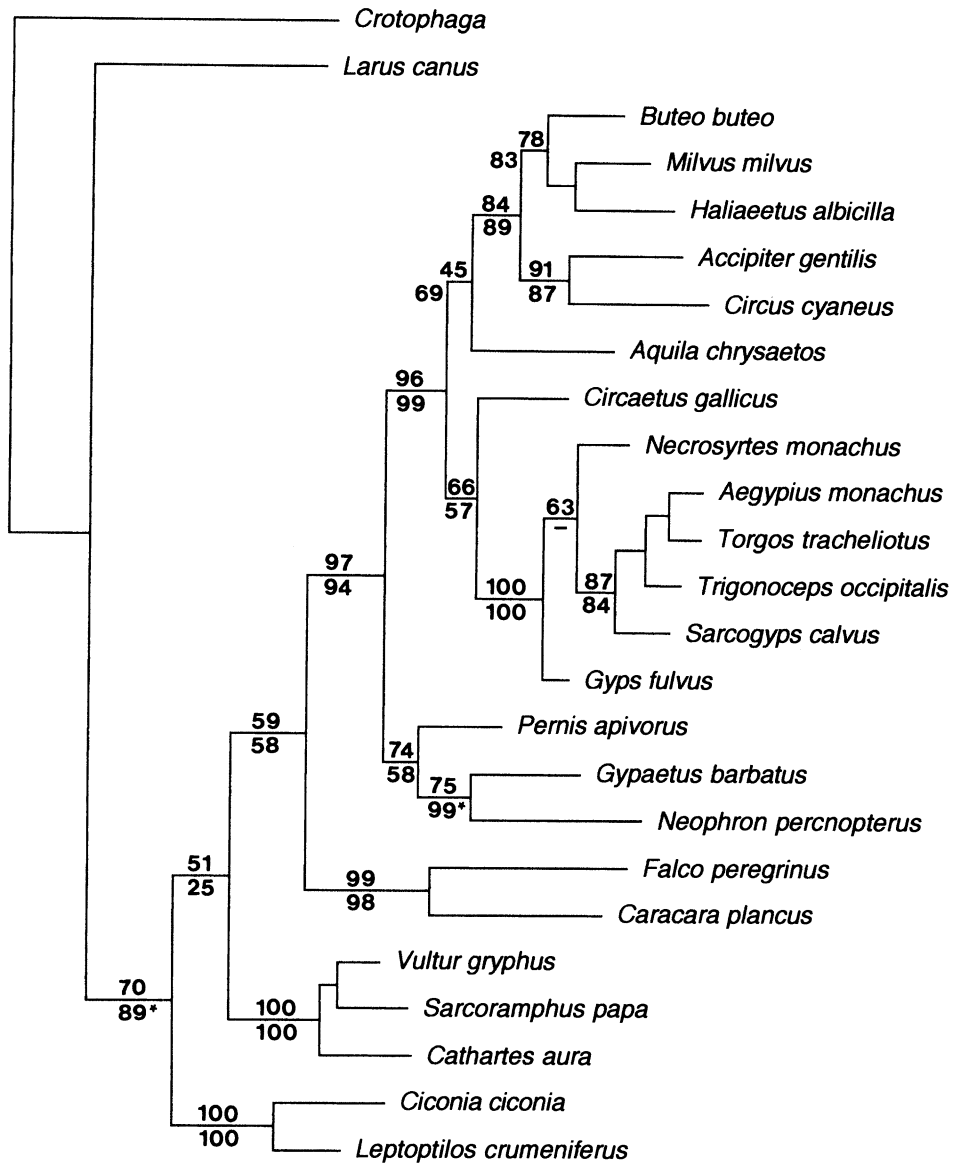

Figure 3. Phylogeny of New and Old World vultures included in this study (except three further *Gyps* species) plus their potential relatives among Accipitridae (8 genera), Ciconiidae (2) and Falconidae (2) based on nucleotide sequence of cyt *b* gene. *Larus canus* (Charadriiformes) and *Crotophaga sulcirostris* (Cuculiformes) were used as outgroups. Branch lengths are proportional to genetic distances. This was the single most parsimonious tree with transversions weighted 5:1 over transitions. Unweighted parsimony yielded a single shortest tree (length 1752 steps) differing only in the placement of *Aquila* (which clustered with Aegypiine vultures). Bootstrap frequencies (100 replicates) are given for parsimony (TV:TS = 5:1; heuristic search, random sequence addition) above branches and for neighbour-joining (Kimura two-parameter distance, TV only) below branches. * Value refers to neighbour-joining with TV + TS; — clade not found by neighbour-joining.

New World vultures are almost as distant from the two storks (range of Kimura distances 15.8–19.1 %) as they are from Old World vultures (range 16.6–20.5 %).

This phylogeny remained stable irrespective of the number (one to three) or combination of outgroups used (*Gallus*, *Crotophaga* and/or *Larus*; not all trees shown). Neighbour-joining analyses with use of Kimura's two-parameter distance (transversions only, to resolve deep branches) yielded identical results with similar bootstrap frequencies (figure 3), except for the position of *Necrosyrtes* (see below).

(*c*) *Relationships among Old World vultures*

Among the Old World vultures we identified the following monophyletic groups (figures 4 and 5); bootstrap frequencies derived from 100 replicates each with parsimony (TV:TS = 5:1) and neighbour-joining (Kimura distance) and various combinations of taxa are given in parentheses:

(1) four members of the genus *Gyps* including the two white-backed vultures ('*Pseudogyps*') (100 %);

(2) four monotypic genera of large, heavy-billed vultures *Aegypius*, *Torgos*, *Trigonoceps* and *Sarcogyps* (weighted parsimony 86 %; neighbour-joining 79–84 %);

(3) *Gypaetus barbatus* and *Neophron percnopterus* (parsimony unweighted 96 %, weighted 69 %; neighbour-joining 99 %);

(4) groups 1 and 2 together formed a well supported monophyletic clade which also included *Necrosyrtes* (100 %).

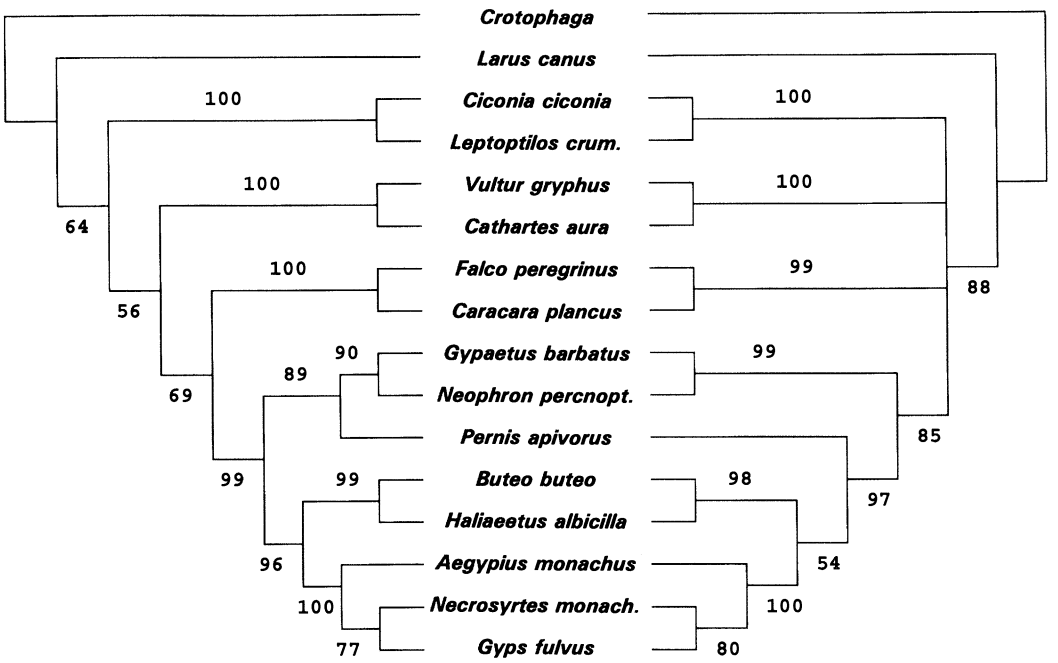

Figure 4. Cladogram with reduced number of derived taxa (compared with figure 3) to test for relationships of *Pernis*, *Gypaetus* and *Neophron*. Left, maximum parsimony (TV:TS = 5:1); right, neighbour-joining tree (Kimura distance, TV + TS). Bootstrap frequencies are based on 100 replicates each. Branches with less than 50% bootstrap support were collapsed. Note different positions of *Pernis* in both trees.

It remained unclear, however, whether *Necrosyrtes* is more closely related to *Gyps* or to the *Aegypius* group (with parsimony its position depended on weighting; neighbour-joining grouped it with *Gyps*).

(*d*) *Are the Old World vultures monophyletic?*

We tested the monophyly of Old World vultures against single representatives of eight other Accipitrid genera (*Buteo*, *Milvus*, *Circus*, *Accipiter*, *Haliaeetus*, *Aquila*, *Circaetus*, *Pernis*) and various combinations of these. This approach also enabled us to suggest the most likely sister taxa of the two Old World vulture clades identified above (*Gyps–Aegypius–Necrosyrtes* and *Gypaetus–Neophron*).

(1) Of the eight non-vulture genera, all except *Pernis* clustered with the *Gyps–Aegypius–Necrosyrtes* clade to the exclusion of *Gypaetus* and *Neophron* (bootstrap support: parsimony 96%, neighbour-joining 99%; figure 3). Imposition of Old World vulture monophyly as a constraint in the (unweighted) parsimony analysis increased the tree length by 12 steps over the most parsimonious tree. These results strongly support a biphyletic origin of the Old World vultures (note that *Gypohierax* was not included and may represent a third independent lineage). Thus booted eagles (*Aquila*, also *Hieraaetus* and *Polemaetus*, data not shown), buzzards (*Buteo*, also *Parabuteo* and *Geranoaetus*), sea-eagles (*Haliaeetus*), *Milvus* kites, *Accipiter* and *Circus* hawks as well as snake eagles (*Circaetus*) all are more closely related to the *Gyps–Aegypius* clade than are *Gypaetus* and *Neophron*.

(2) Among the taxa included, the most likely, though not very strongly supported, sister genus of the *Gyps–Aegypius* clade is *Circaetus* (figures 3 and 5).

(3) In parsimony analyses, *Pernis* clustered consistently as the sister genus of the pair *Gypaetus–Neophron* (maximum parsimony bootstrap 89%; figure 4). Even with the inclusion of other Accipitrids (*Sagittarius*, *Pandion*, *Elanus*; data not shown), this association was never broken. Neighbour-joining analyses were consistent with this result when Kimura distances were calculated by using transversions only (bootstrap 58%). If transitions were included, *Pernis* clustered with the other Accipitrids to the exclusion of *Gypaetus–Neophron* (bootstrap 97%; figure 4). This was the only real conflict encountered between results of parsimony and neighbour-joining analyses.

One may argue that third codon positions are so highly saturated by multiple transitions that they contain little phylogenetic information except among closely related species. We therefore repeated the parsimony analysis with the taxa shown in figure 3 by (1) downweighting transitions at third positions 20:1 (i.e. by their frequency relative to transversions), (2) using first and second codon positions only (cf. Avise *et al.* 1994*b*) and (3) using transversions only (at all codon positions). In general, few taxa changed position in the trees constructed with these options and the results were in full agreement with the conclusions reported in §§3*b–d* (trees not shown). Phylogenetic resolution as judged by bootstrap values did not increase appreciably for any node over the values shown in figure 3.

(*e*) *Genetic differentiation within Aegypiine vultures*

(*i*) *Genus* Gyps

The four species of the genus *Gyps* studied here form a closely related group. Genetic distances (percentage nucleotide divergence) ranged only between 1.0%

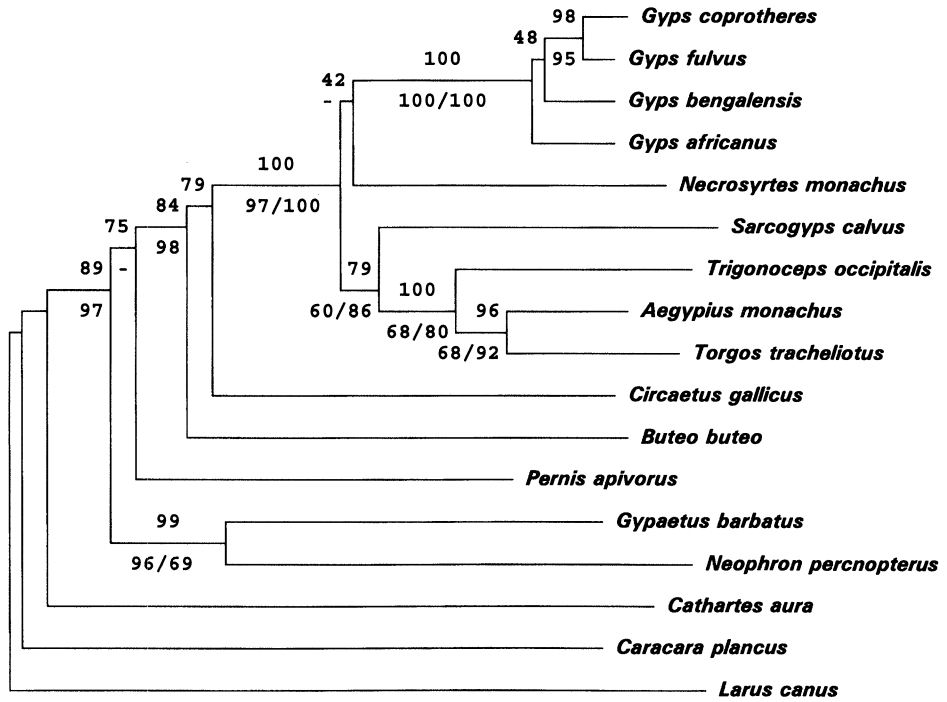

Figure 5. Phylogenetic tree focusing on relationships within Aegypiine 'core group' of Old World vultures. The tree was constructed by neighbour-joining with use of Kimura's two-parameter distance (including TV and TS). Bootstrap frequencies based on 100 replicates each are given as follows. Above branch: neighbour-joining. Below branch: maximum parsimony (PAUP, heuristic search, random sequence addition); unweighted/TV:TS = 5:1 (if only one value is given, it refers to weighted parsimony); – = branch not found in parsimony tree (compare with figure 3).

(*coprotheres* vs *fulvus*) and 2.3% (table 3). This range of differentiation is small compared with other raptor genera (see Seibold 1994) indicating that extant *Gyps* species must have differentiated fairly recently. Within the genus, *fulvus* and *coprotheres* are sister taxa (bootstrap: unweighted parsimony 95%, neighbour-joining 98%) and – given their poor differentiation and allopatric distribution – should be regarded as members of a superspecies. We do not find support for the re-erection of a second genus *Pseudogyps* for the 'white-backed' vultures of Africa (*africanus*) and Asia (*bengalensis*) (*contra* Mundy *et al.* 1992). Genetically, the two white-backed vultures are not closer to each other than each of them is to *coprotheres* or *fulvus* (figure 5, table 3). The phylogenetic reconstruction neither supports nor rejects a sister group relationship between *bengalensis* and *africanus* (bootstrap frequency below 50%).

(*ii*) Aegypius–Torgos *clade*

Members of the four monotypic genera of large vultures *Aegypius*, *Torgos*, *Trigonoceps* and *Sarcogyps* are fairly closely related among each other. The (uncorrected) genetic distances range from 3.9% between *Aegypius* and *Torgos* to 9.1% between *Torgos* and *Sarcogyps*. Phylogenetic analyses (figure 5) support *Aegypius* and *Torgos* as sister taxa (bootstrap 96–100%), but not *Trigonoceps* and *Sarcogyps* (in constrast to morphology-based tree, figure 1). *Sarcogyps* is consistently excluded from the clade comprising the other three species in both parsimony (bootstrap 80%) and neighbour-joining analyses (100%), which indicates that it is the most primitive member of the group and the sister species of the other three.

4. DISCUSSION

(*a*) *Cyt b sequence variation*

The various substitution biases observed in our data set indicate that even synonymous base substitutions may not be entirely neutral, but are restrained by the specific pattern of codon use. Certain substitutions occur much more frequently than others, which leads to 'noise' (loss of phylogenetic information) when analysing relationships of distantly related species. Suppressing such noise and thereby attempting to filter out the remaining phylogenetic signal is legitimate and especially important when investigating phylogenetically old nodes, although methods of how to attain this goal are somewhat controversial. We used parsimony analyses differentially weighted for transitions and transversions. As far as the topology of the resulting trees is concerned, results were not strongly dependent on the weighting scheme, which suggests that the overall topology was fairly robust. However, when asking specific questions about the reliability with which monophyly of certain groups is supported by the data, weighting proved to be useful, if one accepts bootstrap values as indicators of such reliability. When analysing phylogenies that contain both phylogenetically ancient and much more recent nodes, differential weighting is always a compromise between suppressing noise, thus better resolving ancient nodes, and losing signal at younger nodes. We therefore show bootstrap values for different weighting schemes where appropriate (figure 5). Results of parsimony and neighbour-joining reconstructions were in excellent agreement. The only (minor) conflict occurred with the position of *Pernis* relative to other raptors (see §3*d*, figure 4).

(*b*) *Overall relationships of storks, New World vultures and diurnal birds of prey*

Two recent molecular studies have addressed questions relating to the hypotheses tested in this study. One was based on DNA–DNA hybridization (Sibley & Ahlquist 1990); the other (Avise *et al.* 1994*b*) used cytochrome *b* sequences almost as long as in this study and should therefore be directly comparable. There is agreement between both of those studies and ours in that Old World vultures are not part of the stork–Cathartid clade. Other conclusions, however, differ or are not supported by our analysis, as follows.

(1) Sibley & Ahlquist (1990) concluded that New World vultures are the sister group of the storks and that both should rank as subfamilies within the Ciconiidae. Analysis of our cytochrome *b* data does not support this arrangement, since Cathartidae usually clustered as the sister group of diurnal birds of prey (Falconidae + Accipitridae). Although we did not find a clade comprising only storks and Cathartids in any of our analyses, this should not be interpreted as a rejection of the Sibley & Ahlquist arrangement, because bootstrap frequencies for the branch uniting Cathartids with birds of prey were low (25–51% depending on choice of ingroup taxa and outgroups). Probably the relatively variable cytochrome *b* sequences do not contain enough phylogenetic information to resolve these ancient relationships. Longer, somewhat more conservative sequences would be needed instead.

(2) Avise *et al.* (1994*b*) found a clade uniting some storks with New World vultures (including *Vultur*). Within this clade one subgroup, comprising *Mycteria*, *Coragyps*, *Gymnogyps* and *Jabiru*, was supported by bootstrap frequencies of up to 93% (first and second codon positions only), but other Cathartids and other storks were excluded. This implies paraphyly of both Ciconiidae and Cathartidae, a very unlikely proposition. We sequenced only two storks and three Cathartids, but found no evidence for paraphyly of either (sub)family. While Avise *et al.* (1994*b*) conclude that 'the cyt *b* sequences suggest an even closer phylogenetic association between certain storks and New World vultures than was implied by the DNA hybridization data', we find that the three New World vultures we sequenced were almost as distant from the two storks as they were from Old World vultures. Differences between sequences of the same species that we and Avise *et al.* obtained independently were so large (up to 8.2%, see above) that they cannot be regarded as real. Since our data for three of these species were confirmed by studying several individuals of each, we suspect there are problems with the data of Avise *et al.* (confirmed for *Jaribu* by J. Avise, personal communication) and recommend confirmation by studying more individuals.

(*c*) *Polyphyletic origin of Old World vultures*

Although strong morphological and behavioural evidence had indicated otherwise, Sibley & Ahlquist (1990) concluded that, based on DNA–DNA hybridization, 'Old World vultures apparently form a clade' separate from other Accipitrines (including *Accipiter*, *Circus*, *Buteo*) and that 'the Old World vultures are carrion-eating eagles' (p. 485). They did not insist on Old World vulture monophyly, however, because many other Accipitrid genera were not included in their analysis. Our results contradict their conclusions in that *Accipiter*, *Circus* and *Buteo* were found to be more closely related to *Aegypius* and *Gyps* than to *Neophron* and *Gypaetus*.

We conclude that, if the cytochrome *b* gene tree accurately reflects vulture phylogeny, Old World vultures are polyphyletic: *Gypaetus–Neophron* derive from a phylogenetically older ancestor than the rest of the Old World vultures and appear to be more closely related to *Pernis*, long recognized as primitive itself, than to other Accipitridae. The *Gyps–Aegypius* clade (including *Torgos*, *Trigonoceps*, *Sarcogyps*) evolved convergently to the more ancient *Gypaetus* and *Neophron* vultures and shares a much more recent ancestor with *Buteo*, *Aquila* and *Haliaeetus* (and other genera) than it does with *Gypaetus* and *Neophron*. The result is consistent with a number of morphological, karyological, behavioural and other biochemical characters indicating fundamental differences between the two evolutionary lines (see below).

As far as potential sister groups of Old World vultures are concerned, Brown & Amadon (1968) regarded sea eagles as related more closely to vultures than to booted eagles (*Aquila*), *Accipiter* or *Buteo*. This is not in agreement with our phylogeny, which shows sea eagles as members of a clade including booted eagles, *Accipiter* and *Buteo*, but no vultures. Bootstrap support for *Haliaeetus* being closer to *Buteo* than to any vulture reaches 99% (figure 4). Mundy *et al.* (1992) have proposed *Circaetus* and *Terathopius* as sister genera of the *Aegypius–Gyps* clade. This is supported, although not strongly, by our data, which show *Circaetus* as closest to these vultures in both parsimony and neighbour-joining reconstructions. Given the low bootstrap support, it is perhaps likely that other living genera (*Terathopius*?) are even closer to the Aegypiine core group than *Circaetus*.

(*d*) *Relationships among Old World vultures*

The hypothesis that *Gypaetus* and *Neophron* are not closely related to other Old World vultures is supported by the fact that they share characteristics of post-embryonic development and morphology, e.g. strong difference between the dark juvenile and light adult plumages and grasping ability of the foot (reduced in *Gyps*). Compared with other vultures their feeding behaviour is more specialized and includes bone smashing in *Gypaetus* and tool use in *Neophron*. *Gyps* and *Aegypius*, on the other hand, are more generalized carrion feeders. *Gypaetus* and *Neophron* also share surprising similarities in vocalizations, which are very unlike those of other vultures (Thaler *et al.* 1986), and in some display behaviours (Brown & Amadon 1968, p. 308). Since it has not been clearly established which

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of these traits are derived and which are plesiomorphic, they cannot at this point be used to indicate relationships of *Gypaetus–Neophron* within the Accipitridae. The total phenotypic evidence does, however, strongly suggest a closer alliance of *Gypaetus* and *Neophron* with each other than between either of them and other vultures. Jollie (1976/77) interpreted carrion-feeding and a grasping foot as primitive characters among Accipitrids. The Aegypiine core group (*Necrosyrtes*, *Gyps*, *Aegypius* etc.) according to him is well characterized as monophyletic by osteological details and pterylosis. In many respects members of this group resemble *Aquila* eagles more than they do *Gypaetus* and *Neophron*, which differ in many osteological and important pterylotic characters. *Gypaetus* and *Neophron* are assumed by Jollie to represent ancient and independent evolutionary lines, unrelated to the Aegypiine core group. This view is in full accordance with our molecular results. We support the proposal by Mundy *et al.* (1992) to unite them in the subfamily Gypaetinae.

Karyological evidence also supports the distinctness of *Gypaetus* from other vultures, but not that of *Neophron*: *Aegypius*, *Torgos*, *Sarcogyps*, *Gyps* and *Necrosyrtes* have identical karyotypes ($2n = 66$), from which *Gypaetus* differs considerably in the number of chromosomes ($2n = 60$) and their morphology (DeBoer 1976; DeBoer & Sinoo 1984). In a different study, the karyotype of *Neophron* ($2n = 66$) was found to be similar to that of most Aegypiines (Ansari & Kaul 1986). The alternative hypothesis to polyphyly, i.e. uniting all Old World vultures in a monophyletic subfamily Aegypiinae, has been practised or implied by many authors but never received much empirical support. Brown & Amadon (1968), like many others, used this arrangement more for the lack of credible evidence contradicting it than for positive evidence supporting it.

In our analysis *Necrosyrtes* clearly clustered with the *Gyps–Aegypius* clade. It can thus not be viewed as a phylogenetic link between the two convergently evolved lineages of Old World vultures, but is a member of the younger, more derived *Gyps–Aegypius* lineage. Within this group, however, its affinities based on nucleotide sequences remain as unclear as they were with respect to morphology (Jollie 1976/77). White (1950) even advocated uniting *Neophron* and *Necrosyrtes* in a single genus, but this view contradicts all available evidence. *Necrosyrtes* is best regarded as a modern Aegypiine most closely convergent with *Neophron*.

Unfortunately, we were unable to include the palmnut vulture *Gypohierax angolensis* in our study. This species has often been suggested to be related to sea eagles or some other Accipitrid genus rather than being an Old World vulture. It is quite likely that it represents a third independent evolutionary line within the heterogeneous and polyphyletic assemblage of Old World vultures.

(*e*) *Differentiation within the Aegypiine core group*

The close genetic similarity between the four species of *Gyps* studied here reflects their uniform morphology, vocalizations and breeding behaviour (Mundy *et al.* 1992). Based on the rough rate estimate of 2% mtDNA sequence divergence per million years, which seems to apply a wide range of warm-blooded vertebrates (Helm-Bychowski 1984; Wilson *et al.* 1985; Shield & Wilson 1987), these *Gyps* species probably differentiated either within or not long before the Pleistocene. However, the inclusion of other species (*rueppellii*, *indicus*, *himalayensis*) would probably increase the range of differentiation within the genus. This might also reveal stronger evidence for a sister group relationship, not presently supported by our data, between the two white-backed vultures, which share some synapomorphic plumage characters (white back, 12 instead of 14 tail feathers).

The cytochrome *b* divergence values found among the four large, heavy-billed vultures (*Aegypius*, *Torgos*, *Trigonoceps*, *Sarcogyps*) do not exceed the level of differentiation found within several other Accipitriform genera (Seibold 1994), which suggests that it would be justified to unite all four in a single genus *Aegypius*, as has been previously suggested (Amadon 1977). However, to reflect (1) the much closer relationship between *Aegypius* and *Torgos* than between either and *Triginoceps* or *Sarcogyps* and (2) the clearly more primitive position of the Asian *Sarcogyps*, we suggest merging only *Torgos* with *Aegypius* and keeping the other two genera separate.

5. CONCLUSIONS

With regard to the phylogenetic history of birds of prey in general and Old and New World vultures in particular, the results of our analysis are in broad agreement with those of careful morphological studies (especially Ligon (1967) and Jollie (1976/77)), chromosome morphology (DeBoer & Sinoo 1984) and DNA–DNA hybridization (Sibley & Ahlquist 1990). 'Vultures' display fascinating examples of convergent evolution of carrion-eating adaptations. Not only are New and Old World vultures two entirely independent evolutionary lines with some very similar adaptations to a common life style, but also within the Old World vultures there are at least two major independent lines (*Gypaetus–Neophron* and *Aegypiinae*). *Gypohierax*, which was not included here, may well represent a third line. On an even more recent timescale, within the Aegypiines, *Necrosyrtes* has convergently evolved striking similarities in external morphology to the more ancient *Neophron*.

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APPENDIX

Nucleotide sequences of cytochrome b gene of 26 taxa sequenced in this study

The sequences correspond to positions 14995–16020 of the chicken mitochondrial genome (Desjardins & Morais 1990). Dots indicate identity with first line in each block. Unknown nucleotides are marked by '?'.

```
                                 111 111 111 122 222 222 223 333 333 333 444 444 444 455 555 555 556 666 666 666 777 777
                         123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345

 1 Buteo buteo           GGA TCC CTC CTA GGG ATC TGC TTA CTA ACA CAA ATC ATA ACC GGC CTC CTA CTA GCA ACA CAT TAC ACC GCC GAC
 2 Accipiter gentilis    ..C ... ... ... ..A ... ..T C.. ... ... ... ... C.. ... ... ..A ... ... ... ... ..C ... ... ..G ...
 3 Circus cyaneus        ..G ..T ..A ... ..A ... ... C.. ..C ... ... ... C.. ... ... ... ... ... ... .T. ..C ..T T.. ..A ...
 4 Aquila chrysaetos     ..C ... ... ... ..A ... ... C.. A.. ... ... ... C.. ... ... ... ... T.. ..C .T. ..C ... ... ..A ...
 5 Circaetus gallicus    ..G ... ..T ... ..A ... ... C.. ... ..G ... ... C.. ... ... ..A ... ... ... .T. ..C ... ... ..A ..T
 6 Pernis apivorus       ... ... ..A ... ..A ... ... ... A.C ... ..G ... C.. ... ... ..T ... ... ..C ..C ... ... ... ..A ...
 7 Milvus milvus         ... ... ..A ... ..A ... ... C.. ... ... ... ... T.G ... ... ... ... T.. ... ..C ..C ..T ..T ..A ...
 8 Haliaeetus albicil.   ... ... ..A ... ..A ... ... C.. ... ... ... ..T C.G ... ... ... ..G ... ... ... ..C ..T ... ..A ..T
 9 Gypaetus barbatus     ..G ... ..A ... ..A ... ... C.G ..C ... ..G ... C.. ..T ... ... ... ... ..T ..G ..C ... ... ..A ...
10 Neophron percnopt.    ..G ..T ... ... ..A ... ... C.. A.C ... ... ... C.. ..T ... ... T.. ... ..C .T. ..C ... ... ..A ...
11 Necrosyrtes monach.   ... ... ... ... ..A ... ... C.. ... ... ... ... C.. ... ... ... ... ..T ... .T. ..C ... ..T ..A ...
12 Aegypius monachus     ..C ... ... ... ..A ... ... C.. ... ... ... ... C.. ... ... ... ... ... .T. ..C ..T ... ..A ...
13 Sarcogyps calvus      ... ..T ... ... ..A ... ... C.. ... ... ... ... G.. ..T ... ... ... ... ... .T. ..C ... ... ..A ...
14 Torgos tracheliotus   ..G ... ... ... ..A ... ... C.T ... ... ... ... C.. ... ... ... ... ... ..G .T. ..C ... ... ..A ..T
15 Trigonoceps occip.    ... ... ... ... ..A ... ... C.. ... ... ... ... C.. ..T ... ... ... ... ... .T. ..C ... ... ..A ..T
16 Gyps bengalensis      ... ..T ..T ... ..A ... ... C.. ..G ... ... ... T.. ... ... ... ... ... ..T ..G .T. ..C ... ... ..A ...
17 Gyps coprotheres      ..G ..T ..T ... ..A ... ... C.. ..G ... ... ... T.G ... ... ... ... ..T ... .T. ..C ... ... ..A ...
18 Gyps africanus        ..G ..T ..T ... ..A ... ... C.. ..G ... ... ... T.. ... ... ... ... ..T ... .T. ..C ... ... ..A ...
19 Gyps fulvus           ..G ..T ..T ... ..A ... ... C.. ..G ... ... ... T.. ... ... ... ... ..T ... .T. ..C ... ... ..A ...
20 Falco peregrinus      ... ... ..A ... ..A ... ..T C.. GCC ..C ... ... C.. ..T ... ..A T.G ... ..C .T. ..C ... ..A ..G ..T
21 Caracara plancus      ... ..T T.A ... ..T ... ... C.T G.. ..C ... ... C.. ..A ... ..A ..T ... ..C .T. ..C ... ..T ..A ...
22 Vultur gryphus        ... ... ..G ... ..C ... ... C.. A.G ... ... ... C.. ... ... ..A ... ..G ..C .T. ..C ... ... ..G ...
23 Sarcoramphus papa     ... ... ..A ... ..T ... ... C.. A.G ... ... ... C.. ... ..T ..A T.G ..C ..C .TG ..C ... ... ..G ..T
24 Cathartes aura        ... ... ..A ... ..C ... ... C.. G.G ..C ... ... C.. ... ... ... T.A ... ... ..C .T. ... ..T ..T ..A ...
25 Ciconia ciconia       ..C ... ... T.. ..T ... ... C.. AC. .T. ... ... C.. ... ... ..A ..G ... ..C ... ..C ... ... ..A ...
26 Leptoptilos crumen.   ..C ... ... ... ..C ... ... C.. A.. ..C ... ... C.. ..T ... ..A ..G ..G ..T ... ..C ... ... ..A ...

                                 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111 111
   777 788 888 888 889 999 999 999 000 000 000 011 111 111 112 222 222 222 333 333 333 344 444 444 445 555 555 555 666 666
   678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345

 1 ACT ACC CTA GCC TTC TCG TCC GTA GCC CAC ACA TGC CGA AAC GTA CAG TAC GGC TGA CTG ATC CGC AAC TTA CAT GCC AAT GGA GCA TCC
 2 ..C ... ... ... ... ..A ... ... ... ..T ... ... ... ... ... ..A ... ... ... ..A ... ... ..T C.G ... ..T ..C ... ... ...
 3 ... ... ... ... ... ..A ... ..G ... ..T ... ... ... ... ... ..A ... ... ... T.A ... ... ... C.. ... ... ..C ... ... ...
 4 ..C ... ... ... ... ..A ... ..C ... ... ..T ..G ... ... ... ... ..A ... ... ... C.. ... ... ..C ... ... ...
 5 ..A T.. ..G ... ... ..A ... ..T ... ..T ... ..T ... ..T ..G ... ..T ... ... ..A ... ... ... C.. ... ..T ..C ... ... ...
 6 ..A ..A ... ..T ..T ..A ... ..C ... ..T ... ..T ... ... ... ... ... ... ... ..T ... ..T ... C.. ... ... ..C ... ... ..A
 7 ..C ..T ... ... ..T ..A ... ... ... ... ... ..G ... ... ... ..T ... ... ..A ... ... ... C.. ... ... ..C ... ... ...
 8 ..C T.. ... ..T ..T ..A ... ... ... ..T ... ..T ... ... ... ... ..T ... ... ..A ... ... ... C.. ... ... ..C ... ... ..T
 9 ..A G.A ... ... ... ..A ... ..C ... ..T ... ... ... ... ... ... ... ... ... ..A ... ... ... C.. ..C ..T ..C ..C ... ..A
10 ..A G.A ... ... ... ... ... ..T ... ... .T. ... ... G.. ..C ..A ... ... ... T.A ... ... ... C.. ... ..T ..C ... ..C ..A
11 ..A T.. ... ..T ..T ... ... ..T ... ... ... ... ... ... ... ... ... ..T ..A ... ..A ... ..T ... C.. ... ... ..C ... ... ..T
12 ..A T.. ... ... ..T ..A ... ..C ... ..T ... ... ... ... ... ... ..A ... ..A ..G ..A ... ... ... C.G ..C ... ..C ... ... ...
13 ..A T.. ... ... ..T ..A ... ..C ... ... ... ... ... ... ... ... ... ..A ... ..A ..? ... ..T C.. ..C ... ..C ..C ... ...
14 ..A T.. ... ..T ..T ..A ... ..C ... ... ... ..T ... ... ... ... ..A ... ..A ... ..A ... ... C.. ..C ... ..C ... ... ...
15 ..A T.. ... ... ..T ..A ... ..C ... ..T ... ..T ... ... ... ... ... ... ..A ..G ..A ... ... ... C.. ..C ... ... ... ... ...
16 ..A T.. T.. ... ..T ..A ... ..T ..T ... ... ... ... ... ... ... ... ... ..G ... ..A ... ... ... C.. ... ... ..C ... ... ...
17 ..A T.. T.. ... ..T ..A ... ..T ... ... ... ... ... ... ... ... ... ... ..G ... ..A ... ... ... C.. ... ... ..C ... ... ...
18 ..A T.. ... ... ..T ..A ... ..T ..T ... ... ... ... ... ... ... ... ... ?.. ..G ... ..A ... ... ..T C.. ... ... ..C ... ... ...
19 ..A T.. T.. ... ..T ..A ... ..T ..T ... ... ... ... ... ... ... ... ... ?.. ..A ... ..A ... ... ... C.. ... ... ..C ... ... ...
20 ..A ... ... ... ..T ..A ... ..C ... ... ... ..T ... ... ... ... ..A ... ..A ... ..A ... ... ... C.. ... ... ..C ... ... ...
21 ..A .A. ... ... ... ..A ..A ..C T.. ..T ... ... ... ... ... ... ..A ... ... ... ..A ... ... ..T C.. ..C ... ..C ... ..C ..A
22 ..A ... ... ... ... ..A ... ..C ... ... ... ..T ... ... ... ..A ... ... ... ..A ... ... ... C.. ... ..A ..C ... ..T ..A
23 ..A ..T ..C ... ... ..A ... ..C ... ... ... ..T ... ... ... ... ... ... ... ..A ... ... ... C.. ... ..A ... ..G ..T ..A
24 ..A ..A ... ... ..T ... ... ..C ..T ... ... ..T ... ... ... ..A ..T ... ... ..A ... ... ... C.. ... ..A ..C ... ..T ..A
25 ..A ... ..C ... ..T ..A ... ..C ..T ... ... ... ... G.. ... ... ... ... ..G ..A ..T ... ... C.. ... ..A ..C ... ..C ..A
26 ..A ... ..C ..T ... ..A ... ..C ..T ... ..C ... ... G.. ... ... ..T ... ... T.A ... ... ... C.. ... ..A ..C ... ..C ..A

   111 111 111 111 111 111 111 111 111 111 111 122 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222
   666 677 777 777 778 888 888 888 999 999 999 900 000 000 001 111 111 111 222 222 222 233 333 333 334 444 444 444 555 555
   678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345

 1 TTC TTC TTC ATT TGC ATC TAC CTA CAC ATC GGT CGA GGA CTA TAC TAT GGC TCC TAC CTA TAC AAA GAA ACT TGA AAC ACA GGG ATT ATC
 2 ... ... ... ... ... ... ... T.C ... ... ..C ... ... ..T ... ... ... ..A ... ..C ... ... ... ..C ... ... ... ..C ..C ..T
 3 ... ... ... ..C ... ... ... ..C ... ..T ..C ... ..G A.C ... ..C ... ... ... ..G ... ... ... ... ... ... ... ..A ..C ...
 4 ... ... ... ..C ... ..T ... ..T ... ... ..C ... ..G ..C ... ..C ... ..A ... ..T ... ... ..G ..C ... ... ... ..A ..C ..T
 5 ... ... ..T ... .A. ... ... ... ... ... ... ..C ... ..C ... ..C ... ..T ... ... ... ... ... ..C ... ... ... ..A ..C ...
 6 C.. ... ... ..C ..T ... ... ... ... ..T ..C ... ..G ..C ... ..C ... ... ... ... ... ... ..G ..C ... ... ... ..A ..C ...
 7 ... ... ... ..C ... ... ... ... ... ... ..C ... ... ... ... ..C ..T ..A ... T.. ... ... ... ..C ... ..T ... ..A ..C ...
 8 ... ..T ... ..C ..T ..T ... ... ... ..T ..C ... ..G ... ..T ... ... ..A ... ... ..T ... ... ..C ... ... ... ..A ..C ..T
 9 ... ... ... ..C ... ... ... ... ..G ... ..T ..C ... ... ..C ... ... ... ... ..G ... ... ... ... ... ..T ... ..A G.C ...
10 ... ... ... ..C ... ... ... ... ..G ... ... ..C ... ... ..T ... ... ... ... ..G ... T.. ... ... ... ..C ... ..T ... ..A G.C ...
11 ... ... ..T ... ... ... ... ... ..C ... ... ..C ... ..C ..C ... ..C ... ..A ... ... ..T ... ..G ..A ... ... ... ..A ..C ...
12 ... ... ... ... ... ... ... ... ..G ..T ..T ..C ... ..C ..C ... ..C ... ..A ..T ... ..T ... ..G ..C ... ... ... ..A ..C G.A
13 ... ... ... ... ... ... ... ..T ..T ..T ... ..C ... ..T ..C ... ..C ... ..A ... ... ... ... ... ..C ... ... ... ... ..C ...
14 ... ... ... ... ... ... ... ..G ..T ..T ..C ... ..C ..C ... ..C ... ..A ..T ... ..T ... ..G ..C ... ... ... ..A ..C ..T
15 C.. ... ... ... ... ... ... ... ..C ..T ... ... ..C ..C ... ..C ... ..A ..T ... ..T ... ..G ..C ... ..T ... ... ..C G..
16 ... ... ... ... ... ... ... ..G ... ... ... ..C ... ..C ..C ... ..C ... ..A ..T ... ..T ... ..G ..C ... ... ... ..A ... ...
17 ... ... ... ... ... ... ... ... ... ... ... ..C ... ..T ..C ... ..C ... ..A ... ... ..T ... ..G ..C ... ... ... ..A ... ...
18 ... ... ... ... ... ... ... ... ..G ... ... ..C ... ..C ..C ... ... ... ..A ... ... ..T ... ..G ..C ... ... ... ..A ..C ...
19 ... ... ... ... ... ... ... ... ... ... ... ..C ... ..C ..C ... ..C ... ..A ... ... ..T ... ..G ..C ... ... ... ..A ... ...
20 ..A ... ... ..C ..T ... ... A.. ... ..T ..G ... ..C A.T ... ..C ... ..T ..T T.G ..T ... ... ..C ..G ..T ... ..C ..C ...
21 C.T ... ... ..C ... ... ... A.G ... ... ..A ... ..T A.C ... ... ... ... ... ..C ... ... ... ... ... ..C ..C ...
22 ... ... ... ..C ... ... ... ... ..C ... ..T ..A ... ... ..T ... ... ..T ..G ... ... ... ... ..A ... ... ... ..C G.C ...
23 ..T ... ... ..C ... ... ... ... ..C ... ..T ..A ... ... ..T ... ... ... ..A ... ..G ... ... ..G ..? ... ... ... ..T G.. ...
24 ... ... ... ..C ..T ..T ... ... ... ..T ... ..A ... ... ..C ... ... ... ..A ... ..G ... ... ... ..A ... ... ... ..T G.C ...
25 ... ... ... ..C ... ... ... ... ..C ... ..T ..A ... ..C T.C ... ..C ... ... ... ... ... ... ..C ... ... ... ... G.. ...
26 ... ... ... ..C ..T ... ... ..G ... ..T ..A ... ..C T.C ... ..C ... ... ... ..C ... ... ... ..C ... ... ... ..A G.. ..T
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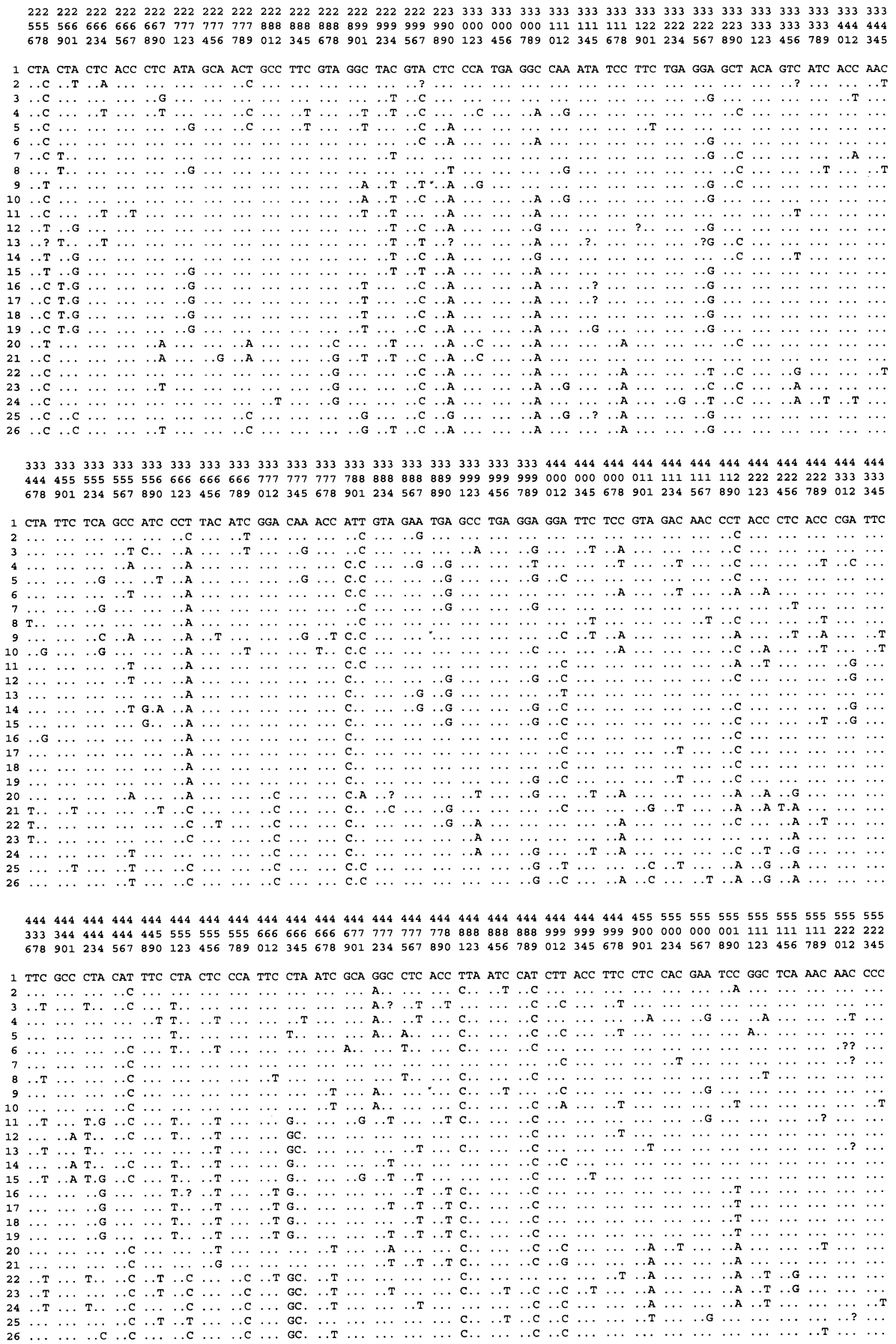

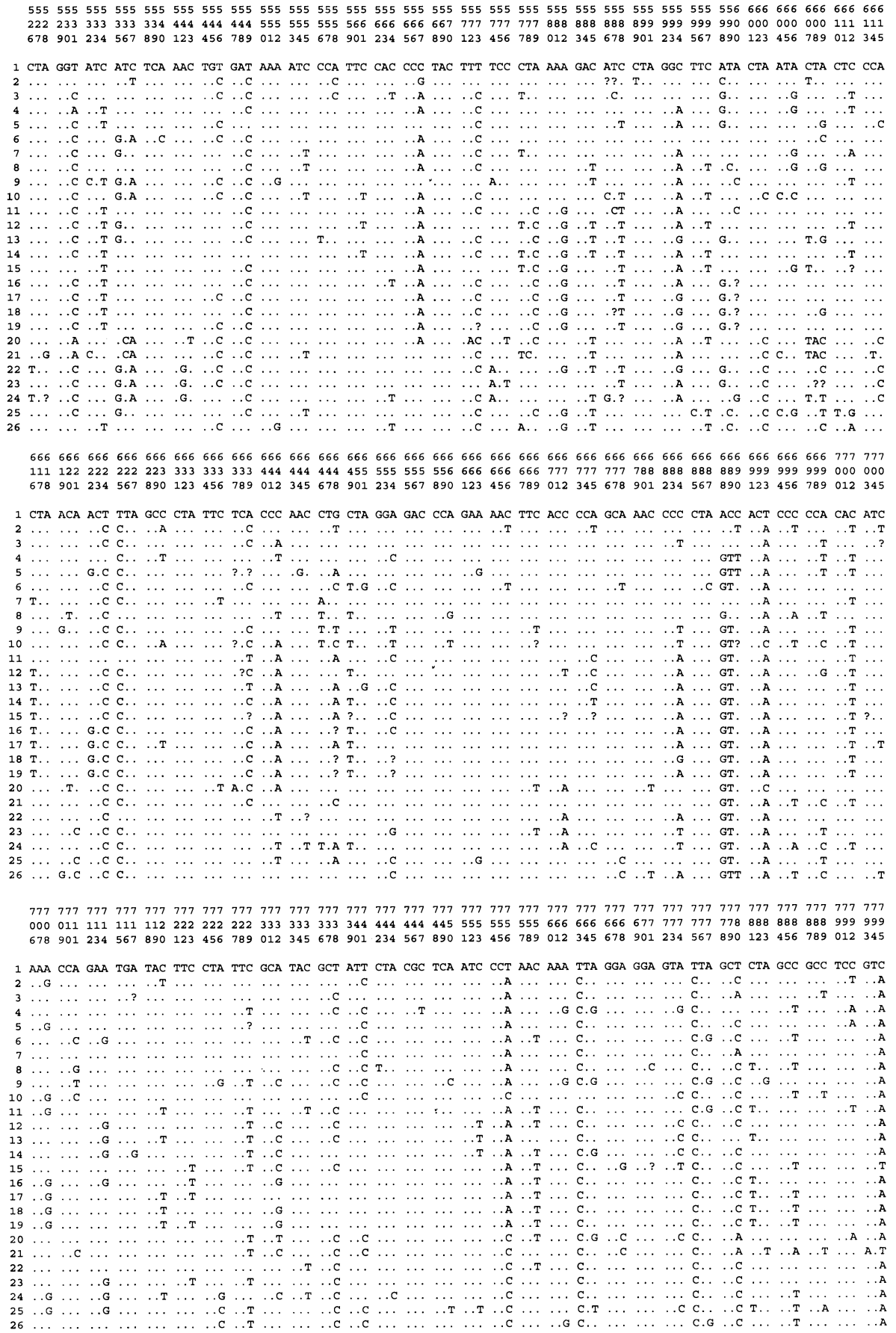

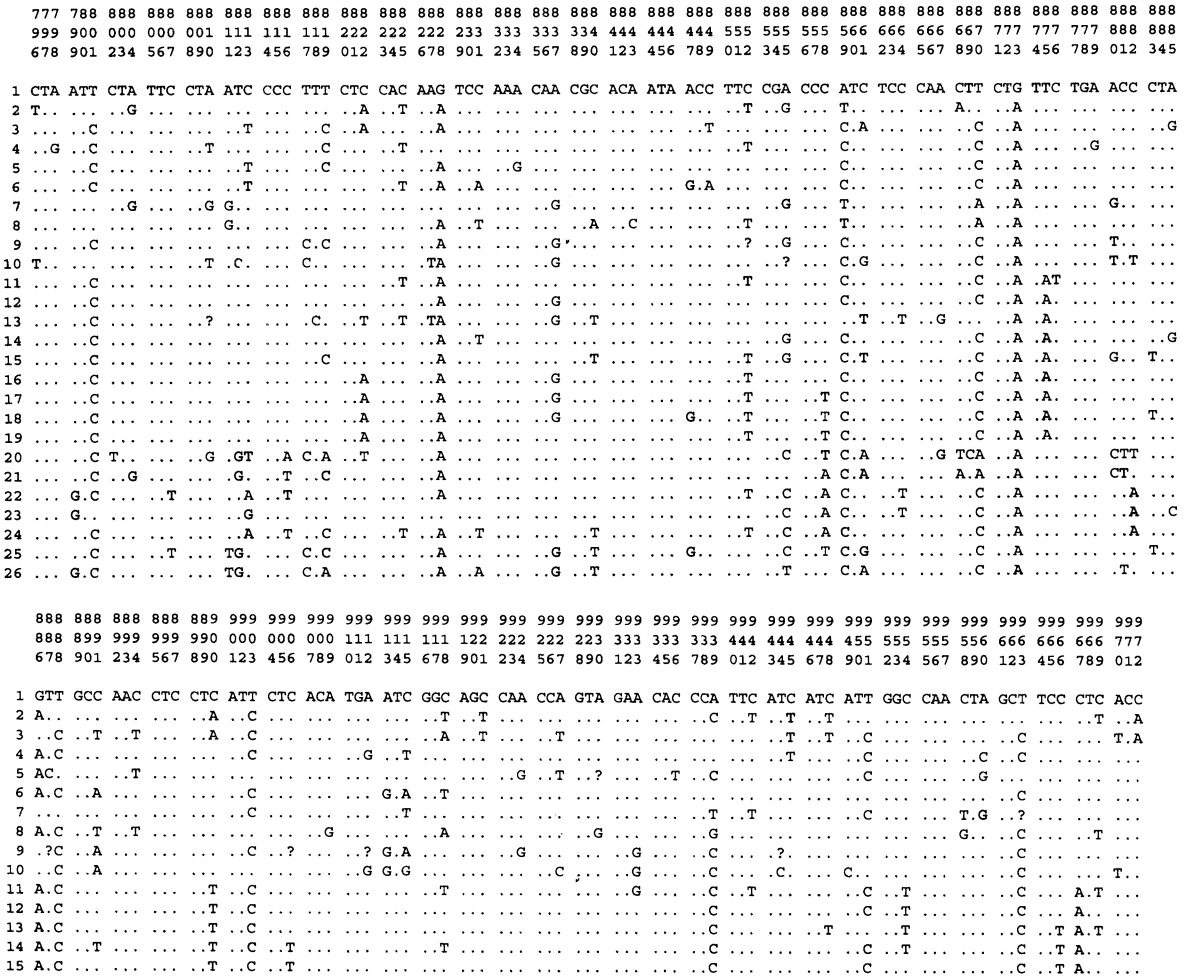

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