Phylogenetics of the common raven complex (*Corvus*: Corvidae) and the utility of ND4, COI and intron 7 of the β-fibrinogen gene in avian molecular systematics

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The common raven (*Corvus corax*) is one of the most widely distributed and recognizable avian species in the world. Recent molecular work, however, described two mitochondrial lineages of the common raven, termed the Holarctic clade and the California clade, and questioned the monophyly of this taxon by placing the Chihuahuan raven (*C. cryptoleucus*) sister to the California clade. We evaluated this phylogenetic hypothesis with additional sequence data and increased taxon sampling. We used ~3.7 kb of DNA sequence data from sections of the mitochondrial coding genes COI, cyt *b* and ND4, a fragment of the non-coding mitochondrial DNA control region, and the entire intron 7 of the nuclear β-fibrinogen gene (β-*fibint* 7). We combined these DNA sequence data to erect hypotheses of relationships for lineages of the common raven and related taxa. Maximum parsimony, maximum likelihood, and Bayesian methods yield a paraphyletic common raven. These analyses nest the Chihuahuan raven within the common raven, with strong support for a sister relationship between the Chihuahuan raven and the California clade. In addition, the pied crow (*C. albus*) is also nested within the common raven, and is sister to the Holarctic clade. Our analyses reveal the challenge of determining phylogenetic relationships and species boundaries in this morphologically conservative genus, and suggest that future molecular work with increased taxon sampling will uncover cryptic species and novel evolutionary relationships. Lastly, this survey is one of a growing number of avian phylogenetic studies to employ either β-*fibint* 7 or COI, and the first to use ND4. We developed a simple procedure for comparing rates of evolution in molecular markers, and show that in *Corvus* the nuclear intron β-*fibint* 7 is evolving at a considerably slower pace than the mitochondrial markers, while COI is evolving at a slower rate than cyt *b*, and ND4 approximately the same rate as cyt *b*. Hence, β-*fibint* 7 and other individual nuclear introns may have limited utility in resolving relationships among recently evolved taxa, whereas both COI and ND4 should be useful in a wide range of avian molecular genetic investigations.

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Introduction

The common raven (*Corvus corax*) is among the most widely distributed and ecologically broad passerines in the world (Madge & Burn 1994; Boarman & Heinrich 1999). Common ravens can be found in nearly every habitat throughout the northern hemisphere, from the deserts of Nevada, to the tundra of Greenland, in remote Siberian forests or dense European cities. In addition, the common raven is one of the most recognizable avian species in the world, familiar to both biologists and laymen because of its conservation impact as a predator of nestling marbled murrelet (*Brachyramphus marmoratus*), California condor (*Gymnogyps californianus*) and other threatened taxa (Boarman & Heinrich 1999), its importance in cognitive and other behavioural studies (Heinrich & Smolker 1998; Heinrich 1999), and its role in the mythology of indigenous cultures as well as modern lore (Heinrich 1989, 1999).

Despite its extensive geographical distribution and diverse habitat preferences, the common raven displays little discrete morphological variation. Several subspecies have been described, but these groupings are clinal (Willett 1941), suggesting that some of the size variation between populations of the common raven is due to Bergmann's rule (Ashton 2002), rather than phyletic or population subdivision. However, a recent phylogeographical survey of the common raven described two deep mitochondrial lineages: a widespread group termed the Holarctic clade, found in Europe, Asia, and North America, and a more restricted group termed the California clade, centred in the south-western USA (Omland *et al.* 2000). No known morphological or behavioural characters diagnose these two groups, yet the Holarctic and California mitochondrial DNA (mtDNA) clades are as divergent, or more divergent, than many recognized avian species pairs (Klicka & Zink 1997; Omland *et al.* 2000). Furthermore, the phylogeny presented by Omland *et al.* (2000) questioned the monophyly of the common raven by placing the Chihuahuan raven (*C. cryptoleucus*) sister to the California clade. However, nodal support for this arrangement was not convincing (73% bootstrap support, Omland *et al.* 2000), probably because the molecular phylogeny was based on two short mitochondrial sequences (~600 bp total) known to be insufficient for resolving relationships among closely related species (Martin *et al.* 1990). Thus, the monophyly and content of the common raven remain open questions.

While the systematics of the approximately 48 species of *Corvus* is still poorly studied (but see Kryukov & Suzuki 2000; Fleischer & McIntosh 2001), the common raven is thought to belong to a small subgroup of *Corvus* we refer to as the 'raven complex'. This complex includes the Chihuahuan raven and several Old World taxa, but does not encompass the four Australian corvids commonly called 'ravens' (Goodwin 1986). The Chihuahuan raven, confined to the arid south-western USA and northern Mexico, differs from the common raven in several subtle ways: the base of the neck feathers are white (hence the specific name); it is smaller with a shorter bill; has a higher pitched call; and is generally more social (Goodwin 1986; Madge & Burn 1994). The remaining members of the raven complex include approximately five large-bodied African and Middle Eastern species with less extensive geographical distributions than that of the common raven. The white-necked raven (*C. albicollis*) is distributed in eastern and southern Africa, and is a robust bird with a powerful bill and prominent white nape. The thick-billed raven (*C. crassirostris*) is endemic to Ethiopia, and bears a close resemblance to the white-necked raven but with a stouter bill and larger body. The fan-tailed raven (*C. rhipidurus*) has a patchy distribution in Saharan Africa and the Middle East, and is a distinctive, all-black species with short rectrix feathers. A more widespread taxon is the brown-necked raven (*C. rufi-*

collis), which has a broad range over Saharan Africa, the Middle East, a portion of central Asia and overlaps with the common raven in northern Africa and central Asia. Interestingly, the brown-necked raven has often been treated as a conspecific with the common raven (Vaurie 1954; Jollie 1978) and can only be distinguished morphologically from the common raven by brownish head and neck feathers and by the structure of the call. The last species in the raven complex, the pied crow (*C. albus*), is widely distributed in sub-Saharan Africa, and has a raven-like profile with a prominent white nape and breast, but exhibits a more crow-like ethology and ecology (Goodwin 1986; Madge & Burn 1994). Importantly, the pied crow is known to hybridize with the brown-necked raven in Ethiopia and Somalia (Ash 1983) and produce fertile offspring (Blair 1961; Alamargot 1987). In addition, the pied crow forms hybrids with the Chihuahuan raven in captivity (T. Fox, pers. comm.). In short, the raven complex includes a small group of large-bodied birds that vary subtly in overall form and plumage, often overlap in habits and habitat, and seem to show some degree of genetic compatibility through examples of hybridization.

While the raven complex includes some of the most intelligent and well-studied avian species (Heinrich 1989, 1999), the only explicit phylogenetic treatments of this group (Jollie 1978; Goodwin 1986) predate DNA sequence data and sophisticated, rigorous phylogenetic methods. It is plausible that the common raven is a widespread species that has repeatedly given rise to more restricted taxa via peripheral isolates speciation (see Mayr 1963; Avise *et al.* 1990; Omland 1997). The paraphyletic relationship recovered by Omland *et al.* (2000) and the deep mitochondrial split between California and Holarctic clades [4% uncorrected cyt *b* sequence divergence; Omland *et al.* 2000] suggest that additional lineages of *Corvus* may branch with one of the two common raven clades.

Our major objective was to use pertinent *Corvus* taxa, additional sequence data from both mitochondrial and nuclear genomes, and model-based phylogenetic methods to better assess the validity of the two common raven clades and the position of the Chihuahuan raven. Our more inclusive sampling will help shed additional light on speciation and diversity within the raven complex. Furthermore, we assessed the phylogenetic utility of two mitochondrial genes ND4 and COI and a nuclear intron [intron 7 of the nuclear β-fibrinogen gene (β-*fibint* 7)] by comparing them with a more commonly used mtDNA marker (cyt *b*).

Materials and methods
Taxon sampling
We collected DNA sequence data from multiple individuals representing divergent subclades from the Holarctic clade, the California clade and the Chihuahuan raven (Appendix).

Table 1 Oligonucleotide primers used to amplify and sequence mitochondrial DNA and nuclear DNA in *Corvus*.

Primer	Marker	Sequence	Reference
HCO 2198	COI	5′-TAA ACT TCA GGG TGA CCA AAA ATC A-3′	Folmer *et al.* (1994)
LCO 1490	COI	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	Folmer *et al.* (1994)
L14841	cyt *b*	5′-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3′	Kocher *et al.* (1989)
H15149*	cyt *b*	5′-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3′	Kocher *et al.* (1989)
L15383*	cyt *b*	5′-GGA CAA ACA CTA GTA GAA TG-3′	Cibois & Pasquet (1999)
H15767	cyt *b*	5′-ATG AAG GGA TGT TCT ACT GGT TG-3′	Edwards *et al.* (1991)
ND4	ND4	5′-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC-3′	Arevalo *et al.* (1994)
Leu	tRNA^{leu}	5′-AC CAC GTT TAG GTT CAT TTT CAT TAC-3′	Arevalo *et al.* (1994)
corII LGL2†	CR	5′-TTT GCA TAC AAT TYT CGT CCA CAT TA-3′	Tarr & Fleischer (1999) (modified)
cor H417†	CR	5′-ACG TYG AGT AGC TCG GTT CTC GTG AG-3′	Tarr (1995) (modified)
FIB-BI7U	β-*fibint 7*	5′-GGA GAA AAC AGG ACA ATG ACA ATT CAC-3′	Prychitko & Moore (1997)
FIB-BI7L	β-*fibint 7*	5′-TCC CCA GTA GTA TCT GCC ATT AGG GTT-3′	Prychitko & Moore (1997)

*Primers H15149 and L15383 were used for sequencing only.
†Ambiguity code: Y = C or T.

We also included one sample each of the African pied crow and white-necked raven. The pied crow and white-necked raven should provide additional polarization to help resolve the position of the Chihuahuan raven and also help to break long branches that might confound our phylogenetic analyses (Felsenstein 1978; Huelsenbeck & Hillis 1993). We were unable to obtain samples of the other members of the raven complex. To root the phylogeny, we used the American crow (*C. brachyrhynchos*) and the rook (*C. frugilegus*), both of which appear distantly related to members of the raven complex (Goodwin 1986; Kryukov & Suzuki 2000; Fleischer & McIntosh 2001). Note that mtDNA for *C. frugilegus* are from Härlid & Arnason (1999), while nuclear DNA (nDNA) for this taxon is from a separate voucher specimen (Appendix).

Laboratory protocols

We isolated and purified genomic DNA from liver and muscle tissue using the DNeasy Tissue Kit (Qiagen Inc.). We amplified fragments of the mitochondrial protein-coding genes COI (658 bp), cyt *b* (925 bp), ND4 and flanking tRNA^{his}, tRNA^{ser} and tRNA^{leu} (825 bp), a portion of the mitochondrial control region (CR) domain I (316 bp), and β-*fibint 7*, as well as small portions of the flanking exons (968 bp) via polymerase chain reaction (PCR) (Saiki *et al.* 1988) using the primers listed in Table 1. We used the following thermal cycle parameters for 25 μL amplification reactions: one cycle of 4 min denature at 95 °C; 35 cycles of 1 min denature at 95 °C, 1 min anneal at 45 °C (COI), 50 °C (cyt *b*), 52 °C (ND4 and β-*fibint 7*), 54 °C (CR), and 1 min extension at 72 °C; one cycle of 4 min extension at 72 °C. We cleaned amplified products using the QIAquick PCR Purification Kit (Qiagen Inc.) and used purified template in 10 μL dideoxy chain-termination reactions (Sanger *et al.* 1977) using ABI Big Dye chemistry (Applied Biosystems Inc.) and the primers in Table 1. Following an isopropanol/ethanol precipitation,

we ran cycle-sequenced products on an ABI 3100 automated sequencer (Applied Biosystems Inc.). All samples were sequenced in both directions.

Sequence analyses

We aligned DNA sequences using the program SEQUENCHER™ 4.1.2 (Gene Codes Corp.) and translated protein-coding nucleotide sequences into amino acid sequences using MACCLADE 4.0 (Maddison & Maddison 2000). In addition, we identified tRNA genes at the 3′ end of ND4 by manually reconstructing their secondary structures following the criteria given by Kumazawa & Nishida (1993). We deposited all DNA sequences in GenBank (Appendix).

To compare rate differences between the five markers, we first computed uncorrected pairwise sequence differences between lineages in PAUP* 4.0b10 (Swofford 2002) for each marker. Then we plotted the p-distances of cyt *b* vs. the p-distances for β-*fibint 7*, COI, ND4 and CR. We then used a one-sample *t*-test (Sokal & Rohlf 1995) to determine whether the regression of each marker vs. cyt *b* deviated significantly from a slope of one (i.e. evolves at a rate significantly different than cyt *b*). This approach should be less sensitive and more accurate than applying a chi-square or Wilcoxon signed-ranks test because it includes information on the magnitude of difference between two markers for each comparison.

Phylogenetic analyses

We used maximum parsimony (MP; Farris 1983), maximum likelihood (ML; Felsenstein 1981) and Bayesian inference (BI; Larget & Simon 1999) phylogenetic methods to infer evolutionary relationships of lineages within *Corvus*. We conducted MP and ML phylogenetic analyses in PAUP* and BI analyses using MRBAYES 3.0b4 (Huelsenbeck & Ronquist 2001).

We executed MP analyses with the branch-and-bound search algorithm (Hendy & Penny 1982) using equally

weighted characters. Most β-*fibint* 7 and CR indels occurred as single base pair substitutions that we coded as fifth character states. However, a 5 bp indel occurred in β-*fibint* 7 between ingroup and outgroup sequences that we treated as a single character (deletion 0, insertion 1) following Prychitko & Moore (2003). We also coded multiple state positions in β-*fibint* 7 as polymorphic. To assess nodal support, we used the bootstrap resampling method (Felsenstein 1985) employing 1000 pseudoreplicates of branch-and-bound searches in PAUP*. Additionally, we calculated branch support (Bremer 1994) for all nodes using the program TREE ROT 2c (Sorenson 1999).

To determine the most appropriate model of DNA substitution for reconstructing relationships of the raven complex under ML, we executed a hierarchical likelihood ratio test (LRT; Felsenstein 1993; Goldman 1993; Yang 1996) in the program MODELTEST 3.06 (Posada & Crandall 1998). The model of DNA evolution that best fit our sequence data was the general time reversible model (GTR; Rodriguez *et al.* 1990) of nucleotide substitution in conjunction with gamma (Γ; Yang 1994a,b) and the proportion of invariant sites (I; Gu *et al.* 1995). We performed ML analyses under the GTR + Γ + I model using the heuristic search algorithm with tree bisection reconnection (TBR) branch swapping and 100 random sequence addition replicates, simultaneously estimating parameter values (with 10 Γ rate categories) and tree topology (i.e. no initial parameter estimates or starting tree). We then successively re-estimated parameter values and searched for trees until we obtained a stable topology and ML score (Wilgenbusch & de Queiroz 2000). We assessed nodal support with 1000 bootstrap pseudoreplicates of fast stepwise-addition searches.

Because MRBAYES 3.0b4 can perform a single phylogenetic analysis using different models of evolution on different data partitions, we performed separate LRTs on each of the five DNA regions. For each of the mitochondrial markers, the most appropriate model of DNA substitution was the Hasegawa, Kishino & Yano model (HKY; Hasegawa *et al.* 1985) with Γ, while the best fit model of evolution for the nuclear intron was the Felsenstein 1981 model (F81; Felsenstein 1981). We then performed BI tree searches, allowing separate parameter estimates (with 10 Γ rate categories) under the chosen models of DNA substitution for each of the five data partitions. We did not specify nucleotide substitution model parameters or a topology a priori. We ran BI analyses for 3×10^6 generations using the default temperature (0.2) with four Markov chains per generation, sampling trees every 10 generations. We plotted log-likelihood scores against generation and calculated summary statistics using TRACER 1.0.1 (Rambaut & Drummond 2003). Lastly, we performed three BI runs to be sure that independent analyses converged on similar log-likelihood scores (Leaché & Reeder 2002).

Estimating divergence times

We used our mtDNA sequence data to estimate the timing of cladogenic events between well-supported lineages of the common raven complex. We excluded β-*fibint* 7 from the molecular clock analysis and subsequent divergence estimates because this nuclear locus is evolving at a markedly different rate from the mitochondrial markers in our system (see Results) and because β-*fibint* 7 lacks a well-calibrated substitution rate.

First we determined whether these mtDNA data follow the assumptions of rate constancy. We executed a hierarchical LRT in MODELTEST to ascertain the most appropriate model of DNA substitution under ML for the combined mtDNA data set. The model of DNA evolution that best fit our mtDNA sequence data was the Tamura–Nei model (TrN; Tamura & Nei 1993) with Γ. We then compared differences in log-likelihood scores for the preferred ML tree optimized with two different, nested models of molecular evolution (TrN + Γ vs. TrN + Γ + molecular clock) using a LRT. In this case, the combined mtDNA data met the assumptions of a rate-constant model because the difference in log-likelihood scores was not significant ($\delta = 12.1775$, $P = 0.20$).

Unfortunately, these corvids lack an adequate fossil record to calibrate a rate of molecular evolution. Therefore, we cannot make an 'internal' calibration (Hillis *et al.* 1996; van Tuinen & Hedges 2001), which would be preferable because generation time, metabolic rate and phylogenetic bias may induce rate heterogeneity between vertebrate taxa (reviewed in Rand 1994; but see Slowinski & Arbogast 1999). Thus, we used two rates calibrated from the same well-characterized geological events for mtDNA from other avian taxa. The first rate, 1.6% pairwise sequence divergence per million years, was calibrated from K2P + Γ corrected cyt *b* sequences (Fleischer *et al.* 1998), and we applied this rate to our cyt *b* data only (Table 2). The second rate of sequence evolution, 3.0% divergence per million years, was calibrated across cyt *b*, ATPase8, and two CR domains using GTR + Γ + I corrected distances (Paxinos *et al.* 2002). We applied this rate to the combined sequences from all four of our mitochondrial regions (Table 2). For both timing estimates, we used these low and high rates with ML corrected pairwise distances, averaged between clades, to date major cladogenic events between well-supported lineages. Regardless, the confidence intervals around molecular clock estimates are extensive (Hillis *et al.* 1996; Graur & Martin 2004), and we interpret our divergence times cautiously.

Results
Genetic variation
Sequences from the protein-coding regions appear functional. In addition, there are no tRNA rearrangements in the data, and the secondary structures of tRNA[his] and tRNA[ser]

Table 2 Pairwise comparisons of mitochondrial DNA sequences among lineages of *Corvus*. Figures above the diagonal denote maximum likelihood (ML) TrN + Γ corrected sequence divergences (%) across all mitochondrial regions, while those below the diagonal represent ML HKY + Γ sequence divergences (%) for cyt *b* only.

	1	2	3	4	5	6	7	8	9	10	11
1 Pied crow (*C. albus*)	—	7.46	5.95	6.03	5.13	5.29	5.29	5.08	5.05	13.72	12.55
2 White-necked raven (*C. albicollis*)	7.06	—	6.76	7.13	5.66	5.82	6.99	7.19	7.41	14.14	13.38
3 Chihuahuan raven (*C. cryptoleucus* NM528)	8.73	7.66	—	0.46	1.56	1.79	5.60	5.53	5.60	13.27	12.18
4 Chihuahuan raven (*C. cryptoleucus* TX376)	9.35	7.94	0.57	—	1.76	1.89	5.77	5.54	5.94	13.88	13.01
5 Common raven (*C. corax* CA168)	8.22	5.91	1.92	2.07	—	0.42	4.73	4.91	4.97	12.54	12.02
6 Common raven (*C. corax* CA176)	7.64	5.44	1.92	2.07	0.22	—	5.02	5.06	5.26	12.98	12.38
7 Common raven (*C. corax* NV2068)	6.21	6.54	6.92	6.65	5.73	5.27	—	0.53	0.69	14.09	11.96
8 Common raven (*C. corax* AK955)	6.21	6.04	5.91	5.67	5.27	4.83	0.45	—	0.61	14.88	12.58
9 Common raven (*C. corax* RUS493)	5.49	6.04	5.91	6.15	5.27	4.83	0.45	0.45	—	13.87	11.70
10 American crow (*C. brachyrhynchos*)	16.42	15.20	16.29	16.78	14.13	15.04	14.14	15.99	14.15	—	14.40
11 Rook (*C. frugilegus*)	16.43	16.69	15.01	16.43	14.41	14.41	14.15	14.60	13.73	21.02	—

Table 3 Proportion of variable, parsimony-informative, and non-synonymous sites in the five DNA markers sequenced.

	cyt *b*	CR	ND4	COI	β-*fibint 7*	Combined
Variable site (%)	15.03	22.47	16.03	13.07	2.38	12.05
Parsimony-informative sites (%)	6.92	9.81	7.79	5.93	0.31	5.25
Non-synonymous sites* (%)	1.84	—	1.18	0.15	—	1.32

*Proportion of sites resulting in an amino acid substitution. Not applicable to non-coding CR and β-*fibint 7*.

are consistent with most other reptile taxa (Kumazawa & Nishida 1993, 1995; Macey & Verma 1997).

Of the 3692 aligned nucleotides, 445 are variable and 194 are parsimony informative. Among ingroup taxa, 263 bp are variable and 123 parsimony informative. Of the 445 variable characters, 86 occur in COI, 139 in cyt *b*, 120 in ND4 (11 from flanking tRNAs), 71 in CR, and 23 in β-*fibint* 7. CR domain I holds the highest proportion of variable and informative sites (Table 3) and displays a faster rate of evolution than cyt *b* (t = 3.1475, P = 0.0027) (Fig. 1). The sequenced fragment of ND4 possesses slightly more variation and phylogenetic content than cyt *b*, but does not appear to be evolving at a significantly different rate than cyt *b* (t = 1.3778, P = 0. 1741). COI demonstrates a slower rate of evolution than cyt *b* (t = −4.3333, P < 0.0001), and β-*fibint* 7 contains the lowest proportion of variable and informative sites and evolves far slower than cyt *b* (t = −60.4667, P << 0.0001).

Phylogenetic relationships

The branch-and-bound equally weighted MP analysis yields a single most-parsimonious tree [length (L) = 608, consistency index (CI) = 0.801, retention index (RI) = 0.715] identical to the tree produced by the ML GTR + Γ + I reconstruction (−ln l = 8092.6331, α = 1.9123, P_{inv} = 0.7571, A–C = 9.6831, G–T = 1, A–G = 61.7612, C–G = 2.8408, A–T = 3.1762, C–T = 102.2105) (Fig. 2). All three partitioned BI analyses converge on the same stable log-likelihood values and separate parameter estimates for the five data partitions (Huelsenbeck & Ronquist 2001). We simply present the

results from the third analysis, which attained stable log-likelihood values within the first 10 000 generations. Because we sampled trees every 10 generations, we discarded the first 1000 trees and retained 299 000 BI trees, which we used to generate a 50% majority rule tree, and for which consensus values represent a group's posterior probability (Huelsenbeck & Ronquist 2001). The summary tree of the nearly 300 000 BI trees (mean –ln l = 8127.5100, σ^2 = 32.4635) has the same topology as the MP and ML trees (Fig. 2). Specific parameter estimates for each marker under the assumed models of evolution in the combined BI analysis are given in Table 4.

In all analyses the common raven is paraphyletic. The Chihuahuan raven and California lineages of the common raven form a monophyletic assemblage in which the Chihuahuan raven forms one clade and California samples form another. Sister to this group is a clade containing the pied crow and the Holarctic lineages of the common raven. However, branch support for this second assemblage varies. Lastly, the white-necked raven is sister to the remaining members of the raven complex, but support for this arrangement is also variable.

As a second test of the paraphyly of the common raven, we constrained the equally weighted, branch-and-bound MP searches to recover only those trees that produce a monophyletic common raven. The shortest tree generated by the constraint MP search is 642 steps long (CI = 0.759, RI = 0.634), 34 steps longer than the most-parsimonious unconstrained estimate of common raven phylogeny. A comparison of the constrained and unconstrained MP topologies using a two-tailed Wilcoxon signed-ranks test (Templeton 1983) suggests
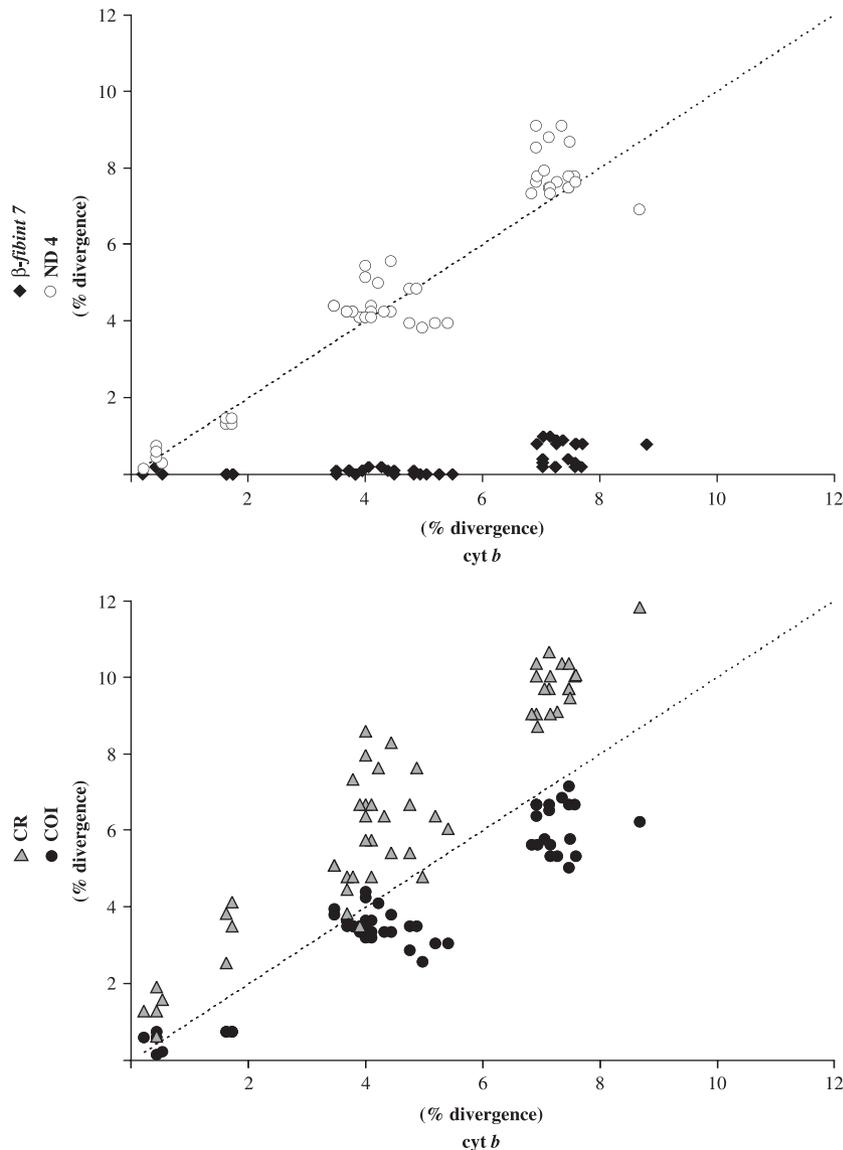
Fig. 1 Scatter diagrams of uncorrected pairwise sequence divergence for cyt *b* vs. COI, CR, ND4 and β-*fibint* 7. The dashed line corresponds to *x* = *y*, for which pairwise divergence values are equal for the two markers being compared. CR displays a faster rate of evolution than cyt *b* ($t = 3.1475$, $P = 0.0027$) and ND4 does not appear to be evolving at a significantly different rate than cyt *b* ($t = 1.3778$, $P = 0.1741$), whereas COI demonstrates a slower rate than cyt *b* ($t = -4.3333$, $P < 0.0001$), as does β-*fibint* 7 ($t = -60.4667$, $P \ll 0.0001$).

that the two hypotheses are incompatible ($P < 0.0001$). Likewise, a constrained ML search under the GTR + Γ + I model produces a single tree ($-\ln l = 8143.5767$, $\alpha = 0.3819$, $P_{inv} = 0.6988$, A–C = 6.6750, G–T = 1, A–G = 53.8772, C–G = 1.1999, A–T = 1.6220, C–T = 89.3658) with a log-likelihood value significantly worse than the unconstrained ML topology using a one-tailed multiple-comparisons LRT (Shimodaira & Hasegawa 1999) with 1000 resampling estimated log livelihoods bootstrap pseudoreplicates ($\delta = 112.7776$, $P = 0.001$).

Divergence times
The LRT could not reject a molecular clock hypothesis ($\delta = 12.1775$, $P = 0.20$). Therefore, we estimated the diver-

gence dates between major clades using the low and high rates of mtDNA evolution of 1.6 and 3.0% sequence divergence per million years (Fleischer *et al.* 1998; Paxinos *et al.* 2002). The average HKY + Γ corrected distance of 6.13% (cyt *b*) and TrN + Γ distance of 6.80% (all mtDNA) between the white-necked raven and the ingroup yields divergence estimates of 3.8–2.3 million years ago. The Holarctic + pied crow clade and the California + Chihuahuan raven clade are separated by 5.58% (cyt *b*) and 5.08% (all mtDNA), suggesting divergences between 3.5 and 1.7 million years ago. However, the average ML distances of 5.68% (cyt *b*) and 5.14% (all mtDNA) between the pied crow and the Holarctic clade produces similar divergence estimates of 3.6–1.7 million years ago. Finally, the Chihuahuan raven and the California clade differ
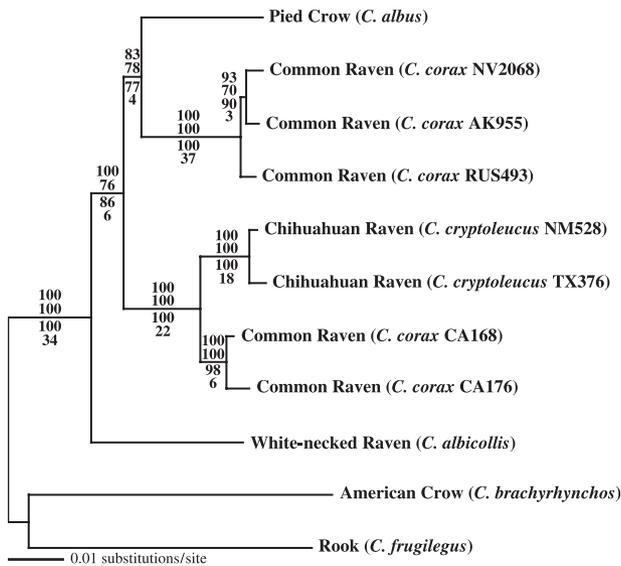
Fig. 2 Phylogenetic hypothesis for *Corvus* lineages. Maximum parsimony (MP; length = 608, consistency index = 0.801, retention index = 0.715), maximum likelihood (ML; GTR + Γ + I: –ln l = 8092.6331, α = 1.9123, P_{inv} = 0.7571, A–C = 9.6831, G–T = 1, A–G = 61.7612, C–G = 2.8408, A–T = 3.1762, C–T = 102.2105) and Bayesian inference (BI; partitioned HKY + Γ and F81: mean –ln l = 8127.5100, σ^2 = 32.4635) phylogenetic analyses yield identical estimates of *Corvus* evolutionary relationships. Locus-specific parameter estimates from the partitioned BI analysis are given in Table 4. Numbers above nodes indicate BI posterior probabilities and ML bootstrap support, respectively, while those below nodes represent MP bootstrap values and decay indices, respectively. Branch lengths are drawn proportional to BI mean estimates of genetic divergence.

by only 1.80% (cyt *b*) and 1.75% (all mtDNA), suggesting divergences between 1.1 and 0.6 million years ago.

Discussion

Phylogenetic relationships

Our data confirm the mitochondrial paraphyly of the common raven. We used 3.7 kb of sequence, from a variety of mitochondrial regions, as well as a nuclear locus, and employed a range of phylogenetic methods, including model-based approaches. The bootstrap support for the California–Chihuahuan node was 100% in both MP and ML analyses, and the BI reliability score was also 100%. Furthermore, we statistically rejected a monophyletic common raven (Shimodaira & Hasegawa test and Templeton test) that would place the Chihuahuan raven outside the common raven. The Chihuahuan raven is clearly sister to the California clade of the common raven. Omland *et al.* (2000) had previously suggested this relationship, but their support for this pairing was weak.

Our DNA data indicate that the common raven includes two ancient lineages (Omland *et al.* 2000), yet microsatellite loci suggest these lineages may be remerging (K. E. Omland, unpublished data). Furthermore, populations throughout much of the western USA contain mixtures of these two mtDNA lineages (Omland *et al.* 2000; K. E. Omland, unpublished data). Another possibility is that the mtDNA data that drive this phylogeny are misleading in this case because random lineage sorting may have fixed two divergent, ancestral haplotypes in different regions of North America. Patterns of species-level polyphyly and paraphyly are widespread among vertebrates, and a number of hypotheses have been proposed to explain such polyphyly and paraphyly (Patton & Smith 1994; Funk & Omland 2003). One commonly invoked hypothesis for molecular paraphyly involves interspecific hybridization (Parham *et al.* 2001). However, recent or on-going hybridization is unlikely to explain this case because the unique haplotypes found in the California, Chihuahuan, and Holarctic clades are quite divergent from each other and always restricted to their respective clades (Omland *et al.* 2000). Nevertheless, ancient hybridization between the common raven and Chihuahuan raven could have resulted in mitochondrial capture and subsequent molecular divergence (Avise 2004), leading to mitochondrial paraphyly of the common raven.

The full taxonomic and evolutionary implications of a paraphyletic common raven are beyond the scope of this paper (detailed mtDNA and microsatellite studies of these issues are underway). One simple taxonomic solution is to elevate

Table 4 Parameter estimates for each marker under the assumed models of DNA substitution in the partitioned Bayesian inference (BI) analysis. The best fit model of DNA evolution was the HKY + Γ model for each mitochondrial region, and F81 for the nuclear locus. Values represent estimated means and variances for the composition of bases (A, C, G, T), unequal rates of substitutions across sites (α), and the transition/transversion ratio (ti/tv) from the nearly 300 000 BI trees.

Marker	A	C	G	T	α	ti/tv
cyt *b*	0.2891 (0.0002)	0.3321 (0.0002)	0.1292 (0.0001)	0.2497 (0.0002)	0.1322 (0.0009)	5.7228 (2.4241)
ND4	0.3273 (0.0003)	0.3024 (0.0002)	0.1301 (0.0001)	0.2403 (0.0002)	0.1931 (0.0030)	12.0938 (23.5772)
COI	0.2754 (0.0003)	0.3108 (0.0003)	0.1596 (0.0002)	0.2542 (0.0003)	0.2413 (0.0099)	7.9322 (11.4175)
CR	0.3729 (0.0007)	0.3446 (0.0006)	0.0972 (0.0002)	0.1853 (0.0004)	0.2990 (0.0134)	5.4504 (3.9088)
β-*fibint 7*	0.3224 (0.0002)	0.1842 (0.0001)	0.1976 (0.0001)	0.2958 (0.0002)	—	—

the California clade of the common raven to create mono-phyletic taxa. However, for reasons discussed above, we feel that species decisions should not be based entirely on these molecular data (primarily mtDNA). Instead, multiple lines of evidence should be used to diagnose distinctness and perman-ence of independent units (de Queiroz 1998). We recognize groups of populations as species if they are monophyletic and possess additional, independent characters suggestive of long-term evolutionary independence. Until we are confid-ent that additional nuclear, morphological, ecological or behavioural characters can further elucidate the two common raven clades, we will refrain from elevating the California clade.

An alternative taxonomic approach is to sink the Chihua-huan raven into the common raven. However, the Chihua-huan raven is distinct in size, shape, and plumage and possesses unique vocalizations and behaviours (Goodwin 1986; Madge & Burn 1994). Our data provide additional evid-ence of the distinctiveness of the Chihuahuan raven; 22–26 unique DNA characters separate the Chihuahuan raven from the California clade. Furthermore, the Chihuahuan raven is not known to hybridize with the common raven, despite the fact that these taxa are broadly sympatric over large areas in south-western USA. Taken together, these observations show that the Chihuahuan raven is clearly diagnosable (Cracraft 1983). Thus, we continue to recognize the Chihuahuan raven as a species, regardless of the complicated systematic issues that may engulf the common raven.

These data also suggest that the African pied crow may be nested within the common raven. All three phylogenetic methods place the pied crow sister to the Holarctic clade, although support values for this relationship vary (MP 78% bootstrap, 4 decay index, ML 78% bootstrap, BI 83% posterior probability). The other African taxon, the white-necked raven, appears to be sister to a clade containing the remainder of the ingroup. Again, support values for this arrangement vary (MP 86% bootstrap, 6 decay index, ML 76% bootstrap, BI 100% posterior probability). Furthermore, short internodes linking both the white-necked raven and the pied crow to the other raven lineages (Fig. 2) suggest a rapid radiation of the entire complex. In fact, average ML corrected sequence divergences (all mtDNA and cyt *b* only) between these four lineages range from 6.80 to 5.08%. Employing low and high rates of mtDNA evolution of 1.6 and 3.0% sequence divergence per million years (Fleischer *et al*. 1998; Paxinos *et al*. 2002) yields divergence estimates between 3.8 and 1.7 million years ago for the four main lineages.

Molecular markers

The field of avian molecular systematics has relied heavily on sequence data from cyt *b* (e.g. Moore & DeFilippis 1997) for both shallow- (e.g. Zink & Blackwell 1998) and deep-level phylogenetic analyses (e.g. Edwards *et al*. 1991). However,

depending on the question and the system, cyt *b* can be an inadequate or inappropriate marker due to known functional constraints (Graybeal 1993, 1994; Meyer 1994; Griffiths 1997). In this study, we sequenced a large portion of cyt *b* as well as fragments of two additional mitochondrial coding genes, COI and ND4. We also sequenced the entire nuclear intron β-*fibint* 7 and portions of the flanking exons. In *Corvus*, COI is evolving at a slower rate than cyt *b*. Several avian studies have demonstrated the conservative nature of COI, which is useful for resolving older bird divergences (Moore & DeFilippis 1997; Cicero & Johnson 2001, 2002; Weibel & Moore 2002a). In contrast, ND4 contains a slightly higher proportion of variable and informative sites than cyt *b*, but is evolving at approximately the same rate. Recently, ND2 has been identified as a useful region of mtDNA that evolves up to 40% more quickly than cyt *b* in certain avian lineages, yet appears less susceptible to saturation (Hackett 1996; Omland *et al*. 1999; Cicero & Johnson 2001, 2002). Although none of our data displays multiple hit problems (based on un-corrected vs. corrected saturation plots for each codon; not shown), we suspect ND4 has similar properties to ND2, and will have greater utility than cyt *b*. ND4 has quickly become the mitochondrial gene of choice in non-avian reptile mole-cular studies, where it is employed to resolve deep-level sys-tematic questions (e.g. Raxworthy *et al*. 2002), conservation issues (e.g. Engstrom *et al*. 2002), and phylogeographical pat-terns (e.g. Feldman & Spicer 2002). Lastly, the nuclear intron β-*fibint* 7 holds the least amount of variation and phylo-genetic signal, and demonstrates a noticeably slower rate of substitution than cyt *b*. In *Corvus*, β-*fibint* 7 could only resolve the oldest split between lineages (outgroup vs. ingroup). Thus, β-*fibint* 7 was unable to recover relationships among our ingroup species, which mtDNA data suggest diverged from each other 3.8–0.6 million years ago. While β-*fibint* 7 was useful in determining relationships among the Picinae (Prychitko & Moore 1997, 2000; Weibel & Moore 2002b), a group thought to have diversified 8–2 million years ago (Pry-chitko & Moore 1997), it was unable to recover intrageneric relationships among *Zenaida* species (Johnson & Clayton 2000). The low substitution rate of β-*fibint* 7 (Prychitko & Moore 1997, 2000; Johnson & Clayton 2000) combined with the long sorting time of this autosomal locus suggest it may be of limited use for resolving shallow avian relationships, but well suited for deep systematic investigations (Prychitko & Moore 2003). We are pursuing the use of multiple nuclear introns in our studies of closely related species in other groups (e.g. *Icterus*; Allen & Omland 2003). Combining data from multiple introns may eventually provide resolution for even closely related lineages, such as in this raven group. Regard-less, we encourage other workers to explore the properties of β-*fibint* 7 and other introns, as well as COI and ND4, to assess their utility as tools for avian species-level phylogenies.

Future studies

By demonstrating that one or more species are nested within the common raven, this study has revealed striking instances of apparent morphological stasis in some lineages (common raven), with subtle (Chihuahuan raven) or even conspicuous (pied crow) morphological changes probably occurring in other lineages. Interestingly, plumage coloration is conservative in the genus; nearly two-thirds of *Corvus* species are completely black (Madge & Burn 1994), often making species identification difficult. This difficulty may hold even for the birds themselves, as there is little evidence of restricted gene flow between the two common raven mtDNA lineages (K. E. Omland, unpublished data). In a similar system, the relatively drab Carolina chickadee (*Poecile carolinensis*) exhibits gene flow between deep mitochondrial lineages (Gill *et al.* 1999). Regardless, the current taxonomy of *Corvus* may seriously underestimate the amount of biological diversity present.

Ravens and crows have been the focus of important studies in a range of biological disciplines (e.g. Heinrich & Marzluff 1995; Hunt 2003) and a complete phylogeny of the genus would facilitate comparative research in a wide range of topics. Given the sizeable content of the genus (~48 species), its nearly cosmopolitan distribution, and a high likelihood that a number of other species also contain cryptic genetic variation, such a project would be a considerable challenge. Detailed morphometric, plumage, mitochondrial and nuclear DNA data are needed. Hopefully, this study provides a starting point for such a project.

Acknowledgements

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Appendix
Specimens used and GenBank accession numbers for DNA sequence data.

Taxon	Locality	Museum number	COI	ND4	CR	cyt *b*	β-*fibint 7*
Corvus albus	Central Region, Ghana	LSUMZ B-39336	AY527232	AY527242	AY527252	AY527262	AY667438
Corvus albicollis	Mulanje Massif, Malawi	MBM 10981	AY527233	AY527243	AY527253	AY527263	AY667439
Corvus corax AK955	North Star Co., Alaska, USA	UWBM 53955	AY527239	AY527249	AY527259	AY527269	AY667445
Corvus corax CA168	Kern Co., California, USA	UCSB 26360	AY527236	AY527246	AY527256	AY527266	AY667442
Corvus corax CA176	Kern Co., California, USA	UCSB 26346	AY527237	AY527247	AY527257	AY527267	AY667443
Corvus corax NV2068	Nye Co., Nevada, USA	MBM 9018	AY527238	AY527248	AY527258	AY527268	AY667444
Corvus corax RUS493	Krasnodarskiy Kray, Russia	UWBM 61493	AY527240	AY527250	AY527260	AY527270	AY667446
Corvus cryptoleucus NM528	Lea Co., New Mexico, USA	AMNH 25323	AY527234	AY527244	AY527254	AY527264	AY667440
Corvus cryptoleucus TX376	Webb Co., Texas, USA	LSUMZ B-37376	AY527235	AY527245	AY527255	AY527265	AY667441
Corvus brachyrhynchos	Montgomery Co., Maryland, USA	No voucher	AY527241	AY527251	AY527261	AY527271	AY667447
*Corvus frugilegus**	Almaty Oblysy, Kazakhstan	UWBM 46364	NC002069	NC002069	NC002069	NC002069	AY667448

*Mitochondrial DNA for *Corvus frugilegus* are from Härlid & Arnason (1999), while nuclear DNA for this taxon are from the above voucher specimen.
AMNH, American Museum of Natural History, New York; LSUMZ, Louisiana State University Museum of Natural Science, Baton Rouge, Louisiana; MBM, Marjorie Barrick Museum of Natural History, University of Nevada Las Vegas; UCSB, Museum of Systematics and Ecology, University of California Santa Barbara; UWBM, University of Washington Burke Museum, Seattle, Washington; AY or NC = GenBank (http://www.ncbi.nlm.nih.gov).