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Dynamics and Phylogenetic Implications of MtDNA Control Region Sequences in New World Jays (Aves: Corvidae)

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Abstract. To study the evolution of mtDNA and the intergeneric relationships of New World Jays (Aves: Corvidae), we sequenced the entire mitochondrial DNA control region (CR) from 21 species representing all genera of New World jays, an Old World jay, crows, and a magpie. Using maximum likelihood methods, we found that both the transition/transversion ratio (κ) and among site rate variation (α) were higher in flanking domains I and II than in the conserved central domain and that the frequency of indels was highest in domain II. Estimates of κ and α were much more influenced by the density of taxon sampling than by alternative optimal tree topologies. We implemented a successive approximation method incorporating these parameters into phylogenetic analysis. In addition we compared our study in detail to a previous study using cytochrome *b* and morphology to examine the effect of taxon sampling, evolutionary rates of genes, and combined data on tree resolution. We found that the particular weighting scheme used had no effect on tree topology and little effect on tree robustness. Taxon sampling had a significant effect on tree robustness but little effect on the topology of the best tree. The CR data set differed nonsignificantly from the tree derived from the cytochrome *b*/morphological data set primarily in the placement of the genus *Gymnorhinus*, which is near the base of the CR tree. However, contrary to conventional taxonomy, the CR data set suggested that blue and black jays (*Cyanocorax sensu lato*) might be paraphyletic and that the brown jay *Psilorhinus* (= *Cyanocorax*) *morio* is the sister group to magpie jays (*Calocitta*), a phylogenetic hypothesis that is likely as parsimonious with regard to nonmolecular characters as monophyly of *Cyanocorax.* The CR tree also suggests that the common ancestor of NWJs was likely a cooperative breeder. Consistent with recent systematic theory, our data suggest that DNA sequences with high substitution rates such as the CR may nonetheless be useful in reconstructing relatively deep phylogenetic nodes in avian groups.

Key words: Control region — Cooperative breeding — Corvidae — Among-site rate variation — Taxon sampling — Combined data — *Psilorhinus morio*

Introduction

The mitochondrial DNA (mtDNA) control region (CR), the major noncoding region of the animal mtDNA molecule, has a role in the replication and transcription of mtDNA molecules (Clayton 1984, 1992). The vertebrate CR is commonly subdivided into three domains that differ from each other in base composition as well as in rate and mode of evolution (Lee et al. 1995; Baker and Marshall 1997). The central domain of the CR, containing the heavy strand's origin of replication, is relatively conserved and is characterized by a high GC content (Saccone et al. 1991). In contrast, the two domains that flank the central domain (domain I and domain II) are typically hypervariable in base substitutions and indels and are characterized by differing base compositions (Saccone et al. 1987; Wenink et al. 1993). Due to the fast rate of

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evolution of domain I and domain II, the CR has been typically deemed to be most appropriate for intraspecific studies (e.g., Vigilant et al. 1991; Edwards 1993; Wenink et al. 1994; Bensch and Hasselquist 1999). It is less well appreciated that the slowly evolving central domain of the CR can resolve phylogenetic relationships at much deeper levels (Saccone et al. 1991; Douzery and Randi 1997). Because few researchers have attempted to use domain I and domain II for resolution of phylogenetic deep branches, little is known about the utility of these domains for such inferences.

In birds, mitochondrial genes that are thought to change more slowly than the CR, such as cytochrome *b* (cytochrome b) and ND2, have traditionally been chosen for phylogeny reconstruction at or above the interspecific level (e.g., De Los Monteros and Cracraft 1997; Voelker and Edwards 1998). In view of the fact that the central domain of the CR may change at a rate similar to or slower than that of "traditional" phylogenetic markers, we were curious about the ability of the entire CR to resolve intergeneric relationships in birds. The categorization of "fast" and "slow" genes in systematics is often vague, and a more useful categorization of genes for phylogenetic inference might focus on the distribution of rates among sites or on the *proportion* of "fast" and "slow" sites per region, with a gene such as cytochrome *b* having roughly one third of its sites in the "fast" category (i.e., third positions; Wakeley 1993; Meyer 1994; Sullivan et al. 1999). Contrary to intuition among many systematists, Yang (1998) proposed that high rates of sequence evolution, in which sequences have experienced on average 0.3–0.6 substitutions per site, may actually be superior for phylogeny reconstruction than slower-changing sites, even in a parsimony analysis. Yang suggests that high rates of evolution will misdirect phylogeny reconstruction due to saturation in base substitutions only at divergence levels much greater than those previously assumed. Faster-changing sites could in fact provide on average more information than slowly changing sites in many cases. We therefore suspected that even the more variable sites of the CR may contain valuable phylogenetic information that could contribute to the resolution of relatively deep branches. Although Yang proposed that high rates of evolution could potentially facilitate tree building, he also cautioned that for noncoding regions, such as the CR, alignment problems could arise at very high levels of divergence. Because previous avian studies show that some sites in the CR are in fact slow-changing sites, such as those in the conserved-sequence-blocks (CSBs; Baker and Marshall 1997), these regions may be particularly useful for phylogenetic resolution above the species level in conjunction with the fast-changing sites.

To further assess the utility of the CR for higher level phylogenetic inferences, a quantitative estimation of the among-site rate variation and substitution patterns in the CR would be useful for a comparison with mtDNA protein coding regions that have been traditionally used in phylogenetics. Some sites in mtDNA protein coding genes, such as third positions, have very high absolute rates of substitution, high transition/transversion (ts/tv) ratios and biased base compositions (Collins et al. 1994; Meyer 1994). These properties can reduce the effective number of character states and increase homoplasy. By contrast, the absolute rates of transitions and the ts/tv ratio of CR domain I sites may be lower, and the base composition more even, than for third position sites of cytochrome *b* in some birds (Edwards 1997). These data suggest a higher level of selective constraint in CR domain I than in third positions of cytochrome b. These characteristics suggest possible increased phylogenetic resolving power for CR domain I sites than for cytochrome b third positions at higher taxonomic levels.

In this study we explored the utility of CR sequences to resolve intergeneric relationships in New World jays (Aves: Corvidae). New World Jays (NWJs) include 7 genera and 36 species that are found throughout North and South America (Goodwin 1976; Madge and Burn 1994). NWJs are well known for their conspicuous sociality and cooperative breeding behavior, as many species have "helpers at the nest" (Brown 1987), and this group has long been a paradigm for studies in evolution and ecology of cooperative behavior and sociobiology (e.g., Brown 1974; Peterson 1992). Nearly half of NWJ species are considered cooperative breeders, and this fraction will likely increase as more information is gathered from poorly studied species. On a statistical basis, NWJs and Corvidae generally appear significantly rich in cooperative breeding behavior in comparison with other bird families (Edwards and Naeem 1993; Arnold and Owens 1998). Cooperative breeding (CB) encompasses a wide diversity of social and breeding systems in different NWJ species and can be temporally sporadic. However, CB behavior is sometimes reported in groups that are closely related to NWJ, such as Old World jays (e.g., *Perisoreus;* Waite and Strickland 1997) and crows (Richner 1990). Thus, as acknowledged by Peterson (1992) and others, having a well-resolved phylogeny may be particularly useful for tracing the evolution of CB and sociality in NWJs.

Although most NWJ species are classified to the genus level without dispute, there is debate among systematists about their intergeneric relationships. Molecular and morphological data sets analyzed independently and/or combined, yield significantly different tree topologies, often with low branch support sets (e.g., Amadon 1944; Hardy 1961, 1969; De Los Monteros and Cracraft 1997; Cibois and Pasquet 1999). For example, De Los Monteros and Cracraft (1997) classified *Aphelocoma* as a sister taxa to *Gymnorhinus,* whereas Amadon (1944), using noncladistic methods, argued that *Gymnorhinus* is basal to all NWJs if it is within the group at all. Follow-

Table 1. Primer sequences, locations, and utility for characterizing New World Jay control regions

Primer	Sequence (5′ to 3′)	Position in data set alignment[a]	Primer versatility/source
Light strand			
PRO.2	AGGAAGAAAGGACTCAAACC	−645	N/A
ND6C	CCGAGACAACCCACGCACAAG	−595	N/A
L16743	TTCTTCGAGATCTACGGCCT	−80	See Tarr (1995)
JCR 01	YGGCCTGAAAAACCATYGTT	−66	General
JCR 03	CCCCCCCATGTTTTTACR	−1	General
JCR 05	GGTAATGCAAGACCTAACCA	101	General
JCR 07	GCCCTATCACTCTCAGGAGC	365	*Corvus*
R1SJ	CTCCCAAGCCAGAGAACCTG	385	*Aphelocoma*
JCR 11	CCTCTGGTTCCTATTTCAGG	523	General
JCR 13	TGTTTTCTTTTTGGGGTCTCTTCAATAAGC	652	*Cyanolyca*
JCR 15	TGCACTTTTACCCCATTCAT	811	General
JCR 17	ATAATGTCATGGTTGCCG	863	*Aphelocoma*
JCR 19	TARCCTAGATTGTCCAAACC	1,006	*Cyanolyca*
JCR 21	TTTAACAAATTTTCATGCGAT	1,129	General
Heavy strand			
JCR 02	GGGTTTACTGTACCTGAAGTGG	205	*Calocitta*
JCR 04	TATGCACAGTRAAACATTCTCG	270	*Calocitta/Corvus*
JCR 06	ACCAGGTTCTCTGGCTTG	405	*Calocitta*
JCR 08	TGAAGCTGGTAGCCGTG	476	General
JCR 10	AATAGGAACCAGAGGCGC	537	General
HD4	CCCGACCAGCTGCATCTGTG	599	See Edwards (1993)
JCR 12	CTGCTACGCACTTGAAGG	699	General
JCR 14	TTACAGACATCAAGCCGCT	758	General
JCR 16	GGAGGTTTAGGTAACAATTCC	929	*Corvus*
JCR 18	TAAATGATTTGGACAATCTAGG	1,030	*Aphelocoma*
JCR 20	TRAAYGYTTTRTRTTTTTRTYTTGY	1,263	General
H1248	CATCTTCAGTGTCATGCT		See Tarr (1995)

[a] Numbers indicate position relative to beginning of domain I (see Fig. 1).

ing on the cytochrome b and morphological analysis of De Los Monteros and Cracraft (1997), we obtained sequence data for 21 NWJs and relatives from the entire control region. To increase resolution of the NWJ tree we sampled several species per genus for the CR sequences. Recent studies suggest that increased taxonomic sampling may improve recovery of higher-level trees, although the importance of increased taxon sampling is debated (Graybeal 1998; Poe and Swofford 1999). In addition to identifying intergeneric phylogenetic relationships, the sequence data itself will be useful for intraspecific studies (for which the CR is so commonly used) in NWJs and related birds (e.g., Brown and Li 1995).

Materials and Methods

DNA Purification and Sequencing. mtDNA extractions were obtained from frozen tissue samples and were purified either by cesium chloride gradients (e.g., Edwards and Wilson 1990) or Promega's Wizard Miniprep DNA Purification system (Beckman et al. 1993) to minimize the possibility of contamination by possible nuclear copies of the mtDNA (i.e., numts; Sorenson and Quinn 1998). Entire or partial CRs were amplified with combinations of L16743 (Tarr 1995), JCR 03, or JCR 01 with H1248 (Tarr 1995; Table 1, Fig. 1). The preliminary corvid CR amplifications for the study were obtained from *Aphelocoma, Cyanocitta, Perisoreus,* and *Corvus* by using the "universal" passerine CR primers designed by Tarr (1995). Products of these primers in conjunction with the primers HD4 (Edwards 1993) and R1SJ (Fig. 1) provided the template for designing the other corvid specific CR primers used in the study.

The mtDNA CR fragments were amplified in 50-μl reactions in a 9600 Perkin Elmer thermocycler using the following thermal conditions: 94°C for 2 min; 94°C for 30 s, 55°C–58°C for 30 s and 72°C for 30 or 60 s, for 35 cycles; 72°C for 7 min. Amplified PCR products were purified with the Qiagen PCR purification kit as described by the manufacturer for use as templates for automated sequencing. Each purified PCR reaction was resuspended in 22–30 μl H_20, and 3–6 μl of concentrated PCR product were used as template along with 4 μl DyeDeoxy Cycle Sequencing reaction mix (ABI Dyeterminator chemistry) and 3 μl of 1 μ*M* solution of a single sequencing primer. A cycle sequencing reaction was then carried out as per manufacturer's instructions (ABI). The sequencing products were purified on a column of Sephadex G-50, electrophoresed, and analyzed on an ABI 373A automated sequencer.

Seventeen individual NWJs representing 14 species and 7 genera were used in the study (Table 2). Outgroup taxa were selected from the family Corvidae: An Old World jay, two American crows, and one yellow-billed magpie. These CRs were sequenced, making for a total data set of 21 corvid individuals (Table 2). The American crows and yellow-billed magpie were specified to root the tree with the NWJs monophyletic, and the Old World jay (*Perisoreus*) was allowed to "float" within the ingroup as an internal test of the phylogenetic signal from the CR data.

Data Analysis. Sequence outputs were assembled using Sequencher v. 4.0 (Gene Codes 1999) to construct the complete CR sequences. Complete CR sequences were then aligned using Clustal W 1.7 (Higgins et al. 1992) using default parameters and subsequent adjustment by

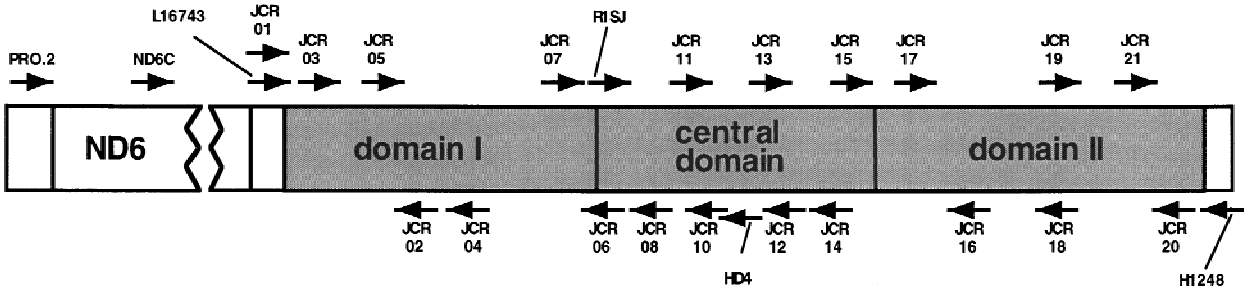

Fig. 1. Schematic diagram of the corvid mitochondrial control region and flanking regions. The control region itself is shaded. Approximate positions of primers used in the study (see table 2) are indicated by arrows.

Table 2. List of species and museum specimens used in study[a]

Genus/species	Common name	Collection	Reference number
New World jays			
Apelocoma			
A. coerulescens	Florida scrub jay	A.T. Peterson	FLSJ 3
A. californica	Western scrub jay	A.T. Peterson	West 2
A. unicolor	Unicolored jay	FMNH	MXJ 451
Cyanocitta			
C. stelleri (1)	Steller's jay	FMNH	ORJ 065
C. stelleri (2)	Steller's jay	FMNH	ORJ 002
C. stelleri (3)	Steller's jay	UWBM	59042
C. cristata	Blue jay	FMNH	GB 072
Cyanocorax			
C. melanocyaneus	Bushy-crested jay	UWBM	56143
C. yncas	Green jay	FMNH	MXJ 396
C. chrysops	Tufted jay	LSUMZ	B18826
Cyanolyca			
C. mirabilis	White-throated jay	FMNH	MXJ 442
C. virideyana	White-colored jay	LSUMZ	B1268
Gymnorhinus			
G. cyanocephalus (1)	Pinyon jay	FMNH	MXJ 283
G. cyanocephalus (2)	Pinyon jay	FMNH	MXJ 282
Calocitta			
C. formosa	White-throated magpie jay	FMNH	MXJ 477
C. colliei	Black-throated magpie jay	FMNH	MXJ 336
Psilorhinus			
P. morio	Brown jay	UWBM	56000
Old World jays			
Perisoreus			
P. infaustus	Siberian jay	UWBM	51672
Outgroups			
Pica			
P. nuttalli	Yellow-billed magpie	FMNH	MXJ 087
Corvus			
C. brachyrhyncus (1)	American crow	UWBM	59089
C. brachyrhyncus (2)	American crow	UWBM	59059

[a] Museum collection sources and specimen catalog numbers are provided. Museum collection abbreviations are as follows: FMNH, Field Museum of Natural History, Chicago, Illinois; UWBM, University of Washington Burke Museum, Seattle, Washington; LSUMZ, Louisiana State University Museum of Natural Science, Baton Rouge, Louisiana.

eye. We were unable to align unambiguously a small segment of variable length (17–43 bp) in the 3′ end of domain II; this region was therefore excluded in the phylogenetic analyses (see Discussion). We obtained ambiguous sequence (~30 bp) that spans from $tRNA^{glu}$ to the center of the structure that is homologous to the chicken's polycytosine hairpin in domain I for all but two taxa, and therefore this region was also excluded from all analyses. The total aligned CR data set used for the analyses spanned 1,298 nucleotide positions. Complete CR sequences have been deposited in the Genbank data base under accession numbers AF218918–AF218938.

To construct trees we implemented a successive approximation method as described in Voelker and Edwards (1998) as follows: maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML) trees were constructed using default parameters in PAUP* version 4.0b (Swofford 1998). These "default" tree topologies were then used to estimate the shape of the gamma distribution parameter (α) to describe among-site rate variation; the transition bias parameter (κ); and the proportion of invariable sites (p_{inv}) under the HKY85 + I + Γ model (Hasegawa et al. 1985; Yang 1998) using empirical base frequencies. Trees were then reconstructed with each of the three tree-

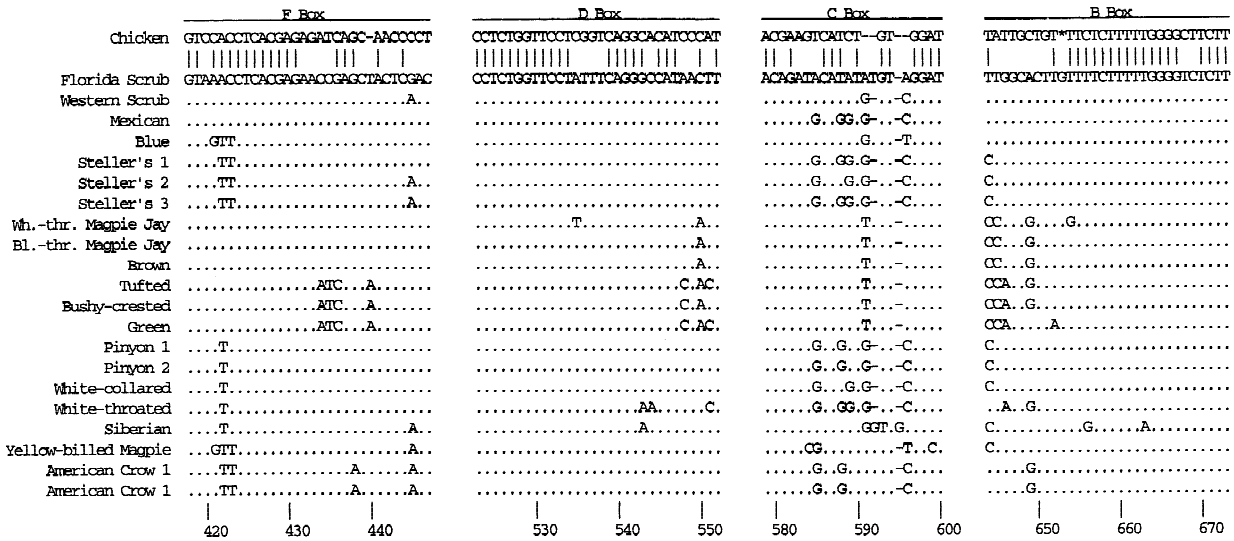

Fig. 2. Alignment of four conserved sequence blocks (CSBs) in the central domains of chicken control region and those of 21 New World Jays and other corvids. Species names and individual number in series when appropriate are indicated at the left (see Table 1). Vertical lines indicate identity of chicken CSB sites and those of *A. coerulescens* (Florida Scrub Jay) individual 1. The nucleotide position in the entire control region alignment is indicated below. (*) in chicken sequence represents indel of nucleotides (TGGTTC).

building methods using one or both parameter values as programs allowed. For example, to incorporate κ into parsimony analyses, we used a step matrix format, and for neighbor-joining a Tamura and Nei (1993) distance was used with a gamma-distribution of rates among sites. The process of estimating parameters and reconstructing new trees based on new estimates was repeated until tree topology stabilized. Because the three domains of the CR displayed different substitution dynamics (see Results), we used two different weighting schemes: one including parameter values for the entire CR, the other employing separate parameters for each of the three CR domains.

For comparison of CR dynamics with cytochrome *b* dynamics we used the NWJ data set from De Los Monteros and Cracraft (1997). This data set included only one species from each NWJ genus and did not include the monotypic genus *Psilorhinus* (often considered congeneric with *Cyanocorax;* see Discussion). Our CR data set included all NWJ species of the cytochrome *b* data set with the exception of the particular species of *Perisoreus* used in the outgroup. We therefore constructed a combined data set for NWJs with one species from each genus which we will refer to as the "combined" data set or, when only a single data partition is used, a data set with a "reduced" number of taxa, to distinguish it from the CR data set that was amassed for our study. The combined data set included 8 taxa and 2,441 nucleotide positions (1,298 nucleotide positions of CR and 1,143 bp of cytochrome *b*). *Aphelocoma coerulescens, Calocitta formosa, Cyanocorax chrysops, Cyanolyca viridicyana, Gymnorhinus cyanocephala,* and *Cyanocitta cristata* (Helm-Bychowski and Cracraft 1993) all include both CR and cytochrome b sequence in the data set (although different museum specimens); *Perisoreus infaustus* CR sequence was concatenated with the cytochrome *b* sequence of *Perisoreus canadensis* since these species are congeneric. CR sequence of *Corvus brachyrhyncus* (1,298 nucleotide positions) was concatenated with 861 bp from cytochrome *b* of *Corvus corone* (GenBank: AF094613). Although both the *Perisoreus* and *Corvus* combined sequence data are technically chimeric, their appropriateness to root the tree is justified because the two pairs of the chimera are in all likelihood more closely related to one another than to those of other species in the data set. We used the combined data set to test for both the effects of combined data analysis and taxon sampling. Finally, a total evidence MP analysis was performed on the data including CR and cytochrome b sequences and the 29 morphological characters as described in De Los Monteros and Cracraft (1997).

To reconstruct ancestral states for cooperative breeding (CB) as a discrete character, we used the parsimony program MacClade (Maddison and Maddison 1992) and Discrete (Pagel 1994, 1999), which provides maximum likelihood estimates of ancestral states assuming either that rates of gain ($0 \rightarrow 1$) and loss ($1 \rightarrow 0$) of CB are similar or that they are different. We used the maximum likelihood tree topology and branch lengths to perform these calculations.

Results

Structure and Base Composition of the Control Region

The NWJ control region has many of the general avian features that have been reported to date in other birds (e.g., Desjardins and Morais 1990; Wenink et al. 1994). The control region is flanked by $tRNA^{glu}$ and $tRNA^{phe}$, consistent with results obtained in some but not all other passerines (Mindell et al. 1998), including *Corvus frugilegus*, whose mtDNA has been completely sequenced (Härlid and Arnason 1999). The length of the corvid CR in this data set ranged from 1,310 to 1,354 bp. Typically the vertebrate CR is subdivided into three domains (domain I, central domain, and domain II) demarcated primarily by different structural features (e.g., CSBs). Accordingly, we designate domain I from the 5′ end of the CR light strand to nucleotide position 400 (~400 bp); the central domain between bp 401–839; and domain II between bp 840 to the 3′ end of the CR light strand (~450 bp). We based our subdivision on the demarcations of Desjardines and Morais (1990) using the aligned comparisons with CSBs from the chicken (Fig. 2). Within the central domain, four conserved sequence blocks (CSBs) were clearly identified: F box, D box, C box, and B box (Fig. 2 and Web site). These CSBs are on average 43% divergent from those found in the chicken sequence. The B box has a 6-bp deletion in comparison to the chicken sequence. We found that there was some correspondence

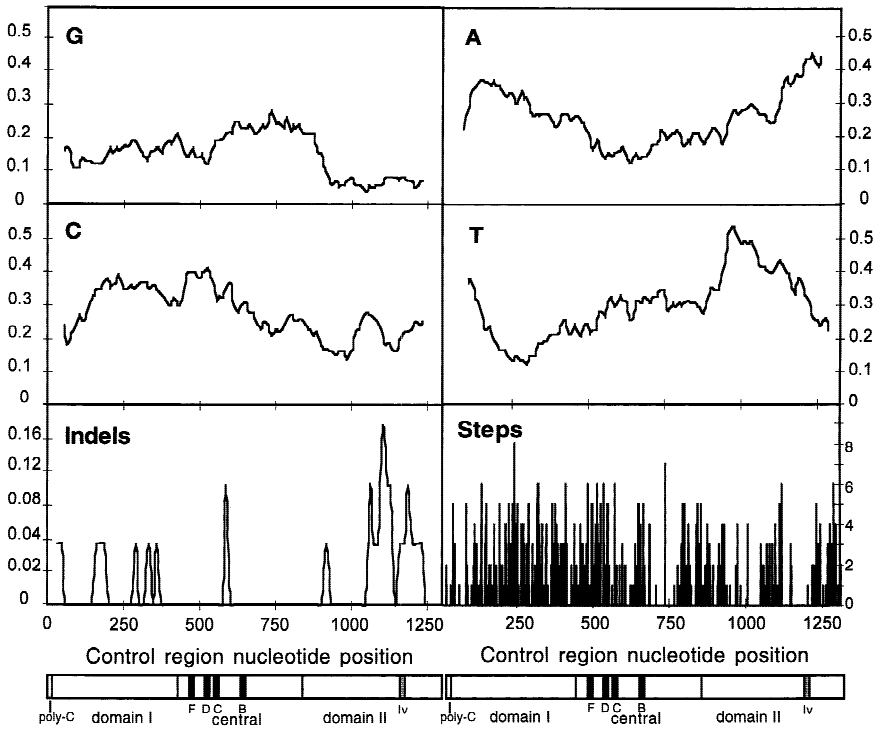

Fig. 3. Frequency of nucleotides and indels and distribution of parsimony steps in the control region (CR) of New World Jays. Conserved sequence blocks in schematic CRs the bottom are indicated in black boxes, the poly-cytosine tract in white boxes and the length-variable region (lv) in grey boxes. For the nucleotide plots the sequence from *Cyanolyca mirabilis.* was used; differences in spatial variation in base composition among species were not substantial. Nucleotide composition was calculated with a sliding window method using a window size of 50 and an offset length of 10. Indel distribution was estimated by comparing two sequences, that from *Cyanolyca mirabilis.* with one from *Aphelocoma coerulescens.* The distribution of parsimony steps per site was calculated using MacClade (Maddison and Maddison 1992).

between base compositional domains in the CR and the domains as defined by sequence blocks. Domain I has a high A and C base composition, while domain II is AT rich (Fig. 3). Frequent thymine stretches ranging 5–8 bp in length and adenosine stretches of up to 6 bp were observed in domain II. A poly-cytosine stretch of sequence that is likely homologous to the chicken hairpin and similar tracts in other mtDNA (Baker and Marshall 1997) is found toward the 5′ end of the corvid CR. Frequent indels are found in domain I and domain II while indels are rare in the central domain (Fig. 3).

Sequence Dynamics and Phylogenies

The average intergeneric sequence difference (uncorrected) among NWJ control regions is 20%, 18%, 9.0%, and 27% for the entire CR, domain I, central region, and domain II, respectively. Pairwise computation of the number of transitions and transversions were performed for the CR and cytochrome *b* data sets. Each data set was partitioned into categories (domain I, central domain, and domain II or by codon position for cytochrome b). The transition/transversion plots for the central domain and domain II are linear (Figs. 4b and 4c), whereas substitution in domain I cluster with no obvious trend and a plateau at about 10% transitions. First and second codon positions in cytochrome *b* (Figs. 4d and 4e) show little variation but a distinctly linear trend, whereas third positions show clustering without a trend and a plateau at about 20% transitions. Domain II had the highest fraction of parsimony informative sites (34%; Table 3).

Phylogenetic trees were constructed for CR data including the 21 corvid taxa using MP, ML, and NJ methods. Initial trees using default settings yielded two different intergeneric relationships. Intergeneric relationships in the ML tree and the NJ tree were identical to each other and to one of the two best MP trees (MP1, length 1,354 steps) (Fig. 5) and differed from the other best parsimony tree (MP2) only in the placement of the genus *Cyanocitta,* the blue jay and Steller's jays. In the most common tree (ML, NJ, and MP1), scrub jays (*Aphelocoma*) comprise the sister clade of *Cyanocitta;* while in the MP2 tree, *Aphelocoma* was basal to the clade containing (*Cyanocitta,* ((*Calocitta, Psilorhinus*), *Cyanocorax*), and *Gymnorhinus*). In addition, minor differences occurred between the three methods in branching pattern within *Cyanocorax* and *Cyanocitta cristata* (see Fig. 5 legend). The monophyly of all groups at the generic level was strongly supported by bootstrap values between 92% and 100%. In addition, the monophyly of all NWJs relative to *Perisoreus* and monophyly of the group of all NWJ genera excluding *Cyanolyca* are well supported. On the NJ tree for the entire CR the maximum divergence between congeneric species was greatest in *Cyanolyca,* where *C. virideyana* and *mirabilis* differ by 18.1%, and less in *Cyanocitta* (10.4%), *Aphelocoma* (9.6%), *Cyanocorax* (9.3%), and *Calocitta* (5.8%).

The number of parsimony steps undergone by CR sites ranged from 0 to 8 on the tree in Fig. 5, including outgroups (Fig. 3). The average number of steps per site on the tree in Fig. 5 was highest in domain II (1.39) and lowest in the central region (0.59). However, the largest

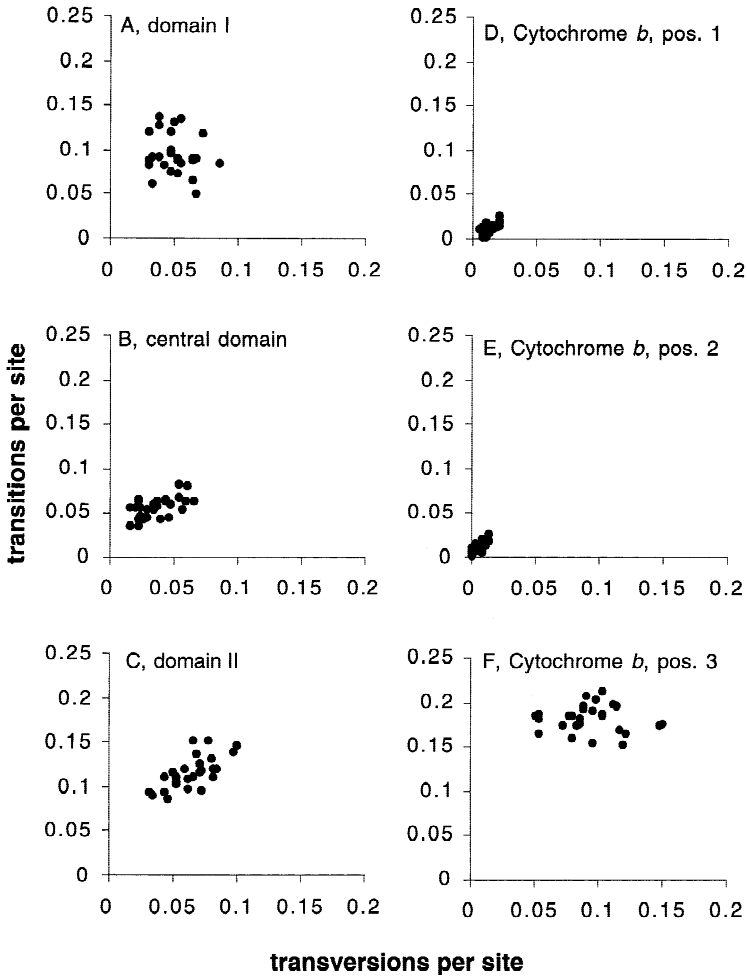

Fig. 4. Pairwise comparisons of transitions and transversions per site for sequences in the reduced data set (1 sequence per genus) for control region sequences (a-c), and cytochrome b sequences (d-f). Cytochrome b data from De Los Monteros and Cracraft (1997). The various data partitions of the control region and cytochrome b are indicated.

number of steps undergone by any site in the central region (7) was almost as high as that in domains I and II (8). The HKY85 + I + Γ model proved a reasonable description of CR substitution dynamics as the likelihoods under the general-time reversible model (GTR + I + Γ model; ~ −7,853.27 to − 7,857.12) were not significantly better than those under the HKY85 + I + Γ model (−7,871.78 to − 7,876.12) via a likelihood ratio test (Huelsenbeck and Crandall 1997). The estimates of κ, α and p_{inv} for the three CR regions under the HKY85 + I + Γ model were very similar for the two equally parsimonious trees (Table 3). In the full CR data set, α was highest for domain II and lowest for the central domain (Table 3). Only the central domain showed substantial among-site rate variation (< 0.5; see Yang 1996). κ values suggested a high transition bias in domains I and II with a lower bias in the central domain (Table 3). The estimated proportion of invariable sites was lowest for domain II and increased for the reduced data set for all data partitions except domain II (Table 3). Overall, α increased and κ decreased in the reduced data set compared to the full data set for CR sequences only. The rank order of α among domains also changed, with the central region having the highest value in the reduced data set. In general the cytochrome *b* gene exhibited higher α (lower among-site rate variation) than the CR, and the transition bias in third positions was highest of all categories. However, the α values of ∞ for first and second positions of cytochrome *b* could be due in part to uncertainty in estimation due to the low amount of variation in these positions (Figs. 4d and 4e).

Successive approximations using the estimated κ and α values for the entire CR and the full data set did not yield any topologies that differed from the default trees except that the MP method converged to a single most parsimonious tree (MP1; Fig. 5) after a single reestimation. In addition, the robustness of the trees was basically unchanged after weighting as indicated by similar bootstrap values with some exceptions. In the reduced data set for the CR data all three tree-building methods yielded the identical topology (((((*Aphelocoma, Cyanocitta*), (*Gymnorhinus,* (*Calocitta, Cyanocorax*)), *Cyanolyca*), *Perisoreus*), crow), although the topologies yielded by the NJ methods were sensitive to the distance method used. The reduced MP and ML trees are consistent with the intergeneric relationships of one of the trees (MP1) based on the full data set. As with the full data set, successive approximations based on estimated κ and α

Table 3. Likelihoods (-ln *L*) and estimates of among-site rate variation (α), transition bias (κ), and proportion of invariable sites (p_{inv}) under the HKY85 + I + Γ model for New World jay control region (CR) and cytochrome *b* sequences estimated on different trees and data sets

Sequence statistics	Entire CR	Domain I	Central Domain	Domain II
Control region—large data set (21 sequences)				
Total sites	1,298	400	459	439
Variable sites	529	178	123	228
Informative sites	417	140	91	186
Uninformative sites	112	38	32	42
Tree 1[a]				
-ln *L*	7,871.78	2,624.87	1,979.18	3,102.91
κ	6.27	7.18	5.07	8.42
p_{inv}	0.45	0.37	0.18	0.32
α	1.29	1.04	0.27	1.51
Tree 2[b]				
-ln *L*	7,876.12	2,629.15	1,978.85	3,103.91
κ	6.28	7.13	5.04	8.54
p_{inv}	0.45	0.37	0.21	0.33
α	1.30	1.04	0.29	1.55
Control region—reduced data set (8 sequences)				
Tree 1[c]				
-ln *L*	5,182.98	1,650.24	1,469.46	1,966.58
κ	5.22	5.59	4.24	7.81
p_{inv}	0.49	0.47	0.67	0.22
α	2.00	4.10	5.50	0.80
Tree 2[c]				
-ln *L*	5,182.99	1,651.91	1,463.96	1,967.19
κ	5.28	5.50	4.24	8.01
p_{inv}	0.48	0.47	0.65	0.24
α	1.81	3.76	3.34	0.84
	Entire cytochrome b	First positions	Second positions	Third positions
Cytochrome *b* (8 sequences)[d]				
Tree 1[c]				
-ln *L*	4,254.29	1,031.43	673.86	2,127.96
κ	7.83	7.68	3.21	45.02
p_{inv}	0.62	0.72	0.77	0.02
α	5.97	∞	∞	1.01
Tree 2[c]				
-ln *L*	4,254.29	1,030.21	673.86	2,127.96
κ	7.83	7.58	3.21	45.02
p_{inv}	0.62	0.72	0.77	0.02
α	5.97	∞	∞	1.01

[a] Tree 1 is the topology of the first MP, NJ, and ML trees; see text.
[b] Tree 2 is the topology of the second (MP2) tree.
[c] Trees for the reduced data sets have the same topology as the corresponding tree of the large data set with the extra taxa removed.
[d] Data from De Los Monteros and Cracraft (1997).

values did not change tree topologies for either CR or cytochrome *b* data sets.

Sources of Phylogenetic Signal

We tested the effect of taxonomic sampling on tree resolution by comparing intergeneric bootstrap values from of the full data set versus the reduced data set. Taxon sampling had a notable effect on the resolution of the intergeneric relationships. In a comparison between the MP trees from the CR data of the two data sets (unweighted), the full data set yielded an average intergeneric bootstrap value of 77% as compared to 59% in the reduced tree data set (Fig. 5). If *Psilorhinus* is considered in intergeneric comparisons in the full data set, the average intergeneric bootstrap value increased to 81%. The effect of reduced taxonomic sampling may also be present in the cytochrome *b* data, where the average intergeneric bootstrap value was only 67% (De Los Monteros and Cracraft 1997).

MP analyses of the reduced data set including the CR, cytochrome *b* and morphological characters combined and separate had primarily affected the placement of *Gymnorhinus* and *Cyanocitta*. The combined CR-cytochrome *b* data (2,441 sites) yielded a single most-parsimonious tree in which the pinyon jay (*Gymnorhinus*) was sister to *Aphelocoma* and *Cyanocitta* was sister to *Cyanocorax/Calocitta/Aphelocoma/Gymnorhinus*.

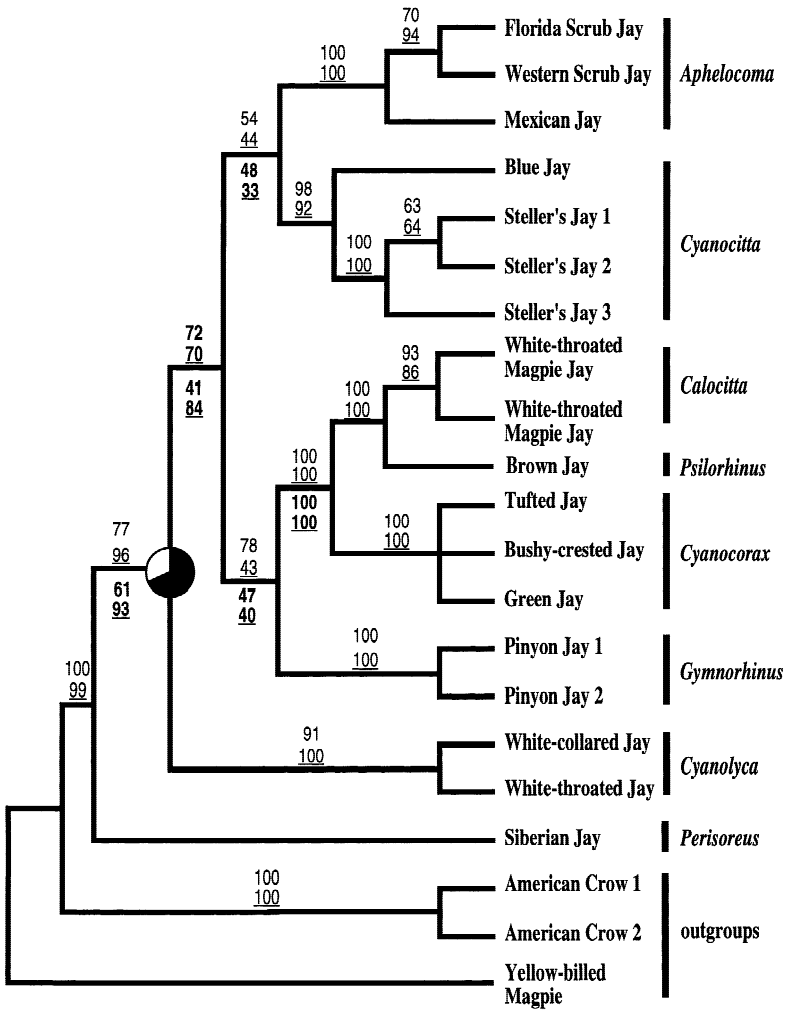

Fig. 5. Phylogenetic tree of 21 corvid individuals based on mtDNA control region sequences (full data set). The consensus tree shown is derived from an unweighted bootstrap parsimony analysis, with the branches within Cyanocorax collapsed at lower than 40% bootstrap support. The pie diagram indicates the chance (68%, black area) that the common ancestor of NWJs was a cooperative breeder under a two-rate model (see text). The two most parsimonious trees (length, 1354 steps) recovered in heuristic searches both placed *Cyanocorax yncas* and *C. melanocyaneus* as sister species, but differed in the placement of Aphelocoma (see text). The NJ tree (see text) differed in placing *C. yncas* and *C. chrysops* as sister taxa, whereas the ML tree was similar to the MP trees with regard to Cyanocorax but placed Steller's Jays 1 and 3 as sisters rather than 1 and 2. The two bootstrap percentages out of 1000 replicates above all internal branches indicate the results of unweighted and weighted (underlined) parsimony analyses. The two additional bootstrap percentages below intergeneric branches (also out of 1000 replicates) indicate results from reduced CR data analysis and CR/cytochrome *b* combined analysis (underlined).

Nonetheless, the consensus bootstrap parsimony tree of the combined molecular data yielded a tree with intergeneric relationships as in Fig. 5. The average intergeneric bootstrap support was greater with the combined molecular data set (73%) than with the CR data alone (59%; Fig. 5). De Los Monteros and Cracraft (1997) showed that the cytochrome *b* data alone yielded the same topology as the combined cytochrome *b*–morphology data set, but that extra-morphological characters increased bootstrap values. As in the single heuristic search tree of combined molecular data, this tree also implied *Gymnorhinus* as a sister to *Aphelocoma*. A parsimony analysis of total evidence data (CR, cytochrome *b*, and morphological characters) yielded a tree topology that placed *Gymnorhinus* as basal to the *Aphelocoma-Cyanocitta* clade, a topology that is intermediate between the CR tree (Fig. 5) and that produced by cytochrome *b* and morphology. Despite the differences in these reduced data-set tree topologies, none of the differences between molecular data sets were statistically significant as analyzed with Templeton's test (Templeton 1983). The CR data on the reduced CR tree (897 steps) was only 10 steps shorter than on the cytochrome *b* tree, and the cytochrome *b* data on the cytochrome *b* tree (642 steps) was only 5 steps shorter than on the CR tree (both tests, $p > 0.05$).

To study the evolution of cooperative breeding, we rather simplistically coded all clades except yellow-billed magpie, western scrub jay, and *Cyanocitta* as cooperative (state 1). Under this scoring scheme, MacClade predicted that the ancestral state for NWJs was cooperative. A maximum likelihood analysis using Discrete suggested that a model in which rates of forward and backward character change were unequal ($0 \rightarrow 1 = 0.021$, $1 \rightarrow 0 = 0.008$, $-\ln L = 19.267$) was superior to a model in which character change was equal ($0 \rightarrow 1 = 1 \rightarrow 0 = 0.0118$, $-\ln L = 22.561$). Under the two-rate model, the probability that the ancestor of NWJs was cooperative was estimated to be 68% (59% under the one-rate model).

Discussion

The complete CR sequences appear to be useful for resolving intergeneric relationships in New World jays. A

number of factors seem to lend credibility to the signal that we see from the CR data. Multiple species were sampled from each genus except for the monotypic brown jay, *Psilorhinus.* Our analyses indicate that taxon sampling did improve phylogenetic signal somewhat, particularly at deeper nodes in the tree: average intergeneric bootstrap support was lower when only a single exemplar per genus was sampled. By sampling multiple species per genus, we were also able to judge how well our data set delimited these higher categories: We found that all traditionally recognized genera of New World jays were supported by our data except for *Psilorhinus* (= *Cyanocorax, sensu lato*), which clustered with *Calocitta* instead of the genus to which it is usually allied, *Cyanocorax.* In addition, the Old World jay exemplar (Siberian jay; *Perisoreus*) was always basal to the NWJs when the tree was rooted with the other three outgroup taxa. Finally, a previously published cytochrome *b* data set and our CR data were not statistically incongruent with each other. These points support the idea that fast evolving DNA sequences such as those of the CR may be valuable in relatively deep phylogeny reconstruction and with divergences as high as 20% as long as alignment is satisfactory. Fast-evolving CR sequences will also be useful for uncovering the unexpectedly high diversity within some clades, such as *Cyanolyca,* where divergences exceeded 18%. Nonetheless, two taxa, *Gymnorhinus* (the pinyon jay) and *Cyanocitta* (Steller's and blue jays) consistently shifted in our trees, and, overall, bootstrap support for the affiliations of these taxa was low.

Taxon sampling seemed to have a much more visible affect on estimates of sequence dynamics than tree topology. These results are consistent with results of other recent studies exploring this issue (Sullivan et al. 1996, 1999; Edwards 1997; Voelker and Edwards 1998; Excoffer and Yang 1999). All estimated values of α increased in the reduced data set estimation with the exception of domain II. The increase in α may be expected if much of the signal for among-site variation in the data set comes from closely related sequences, especially when more than two species per genus are included in analyses. The decrease in the value of for domain II was less anticipated. The cause of this pattern may be attributable in part to the high rate of evolution of this domain, which exhibits the greatest average divergence of the CR. The apparent differences in substitution dynamics between large and small data sets may, however, simply be due to sampling error (Sullivan et al. 1999). Sullivan et al. (1999) suggest that substitution dynamics can be reasonably estimated with about 20 sequences in the data set, a condition that is met by our full data set.

Although the weighting schemes and sequence models did not seem to have a great effect on tree topologies, they nonetheless provided valuable insight into the mode of evolution of the CR. Our data suggests that the evolutionary dynamics of domains I and II, (transition bias, κ, and among-site rate variation, α), are broadly similar to one another and distinct from those in the central region. This conclusion does not necessarily follow from the common observation that the average substitution rate in these two partitions are drastically different; differences in average substitution rates do not necessarily imply differences in substitution dynamics. The CSBs in the conserved central domain may be the basis for the highest level of among-site rate variation (lowest α). A conserved block found in the expected position of CSB-1 in the central domain is conserved among corvids although it shares only seven consecutive sites with the 27 bp of CSB-1 of the chicken. A CSB has been reported in other avian CRs in domain II (i.e., CSB-1: Quinn and Wilson 1993) but was not identified unambiguously in the NWJ CRs. Our data (e.g., Figs. 3 and 4) also suggest an association between transition bias and equilibrium base composition, a relationship that has been noted before (Collins et al. 1994; Perna and Kocher 1995). For example, the three CR domains are reasonably reflected in the spatial changes in base composition across the CR. The comparison of dynamics of CR and cytochrome *b* imply that saturation of transition is less of a problem in the CR data than in the third positions of cytochrome *b,* which have an extremely high transition bias (Edwards 1997).

The intergeneric relationships derived from these data are in general concordance with a previous molecular phylogeny for this family (De Los Monteros and Cracraft 1997) with the exception of a single node. Moreover, what differences exist between the cytochrome *b*/morphology tree and the CR tree are not statistically distinguishable by either data set. The primary difference between the cytochrome *b* tree and the CR or the combined CR–cytochrome *b* trees is in the placement of *Gymnorhinus,* a morphologically highly derived taxon (Amadon 1944). The differential placement of *Gymnorhinus* in the two molecular data sets may be due to homoplasy or other molecular artifacts, and thus the placement of this taxon remains unresolved. *Cyanocitta* also shifted positions, either after incoporating a weighting scheme derived from the CR data or when the CR and cytochrome *b* data were combined. Indeed, we were surprised that our CR data could not discriminate between the placements of *Gymnorhinus* and *Cyanocitta* given the reasonably high bootstrap values for several intergeneric nodes between the two positions at the base and near the tip of the CR tree. The inability of over 2 kb of mtDNA to solidly resolve generic relationships in NWJs may indicate that these genera diverged in rapid succession, resulting in short internodes (see Walsh et al. 1999).

Our CR data set included a species that was absent in the cytochrome *b* study—the brown jay (*Psilorhinus morio*). The generic classification of the brown jay is in dispute as some researchers consider this species as *Cya-*

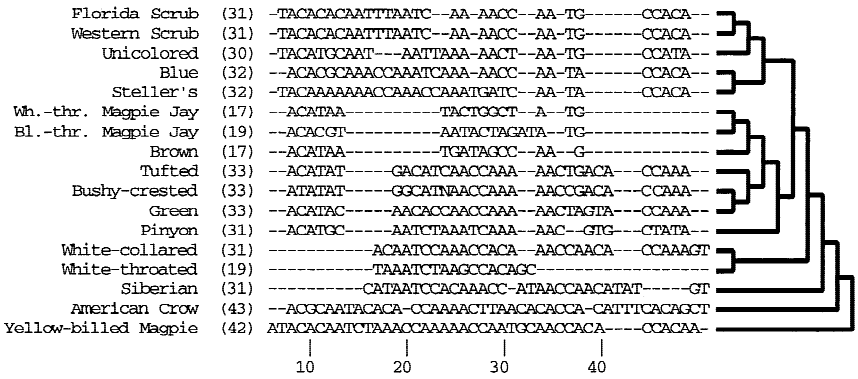

Fig. 6. Manual alignment of the length-variable region of domain II of New World Jay and corvid control regions. Common names of Jays can be linked with Latin names in Table 1. In parentheses beside each name is the total length of the region for that species. The region occurs after position 1188 in the Florida Scrub Jay CR and after position 1159 in the Yellow-billed Magpie CR, or at position after position 1210 in the aligned NWJ sequences. A schematic of the tree depicted in Figure 5 appears at right.

nocorax morio (e.g., Williams et al. 1994), while other researches consider this species to form a monotypic genus, *Psilorhinus* (e.g., Haemig 1989). Surprisingly, our data strongly suggest that *Psilorhinus* in fact lies outside of the *Cyanocorax* clade and is sister to *Calocitta.* The case of *Psilorhinus* represents a good example of the possible paraphyly of genera erected on morphological grounds and in the absence of tree thinking by traditional alpha-taxonomy, which naturally assumes each named genus (*sensu lato*) to be monophyletic. *Psilorhinus* may in fact be morphologically more similar to *Cyanocorax,* yet this similarity does not guarantee monophyly of the clade *Psilorhinus-Cyanocorax* with respect to sister genera. Paraphyly of *Cyanorax* may be as parsimonious for many nonmolecular characters as monopohyly; in this scenario, *Calocitta* and *Psilorhinus* both represent derived *Cyanocorax.*

Despite the difficulty of aligning the length-variable region of domain II across jay species (Fig. 6), the absolute length of the region nonetheless appears to provide some insight into phylogenetic relationships. For example, within genera the lengths of this region are highly consistent, except for *Cyanolyca* species, perhaps because *Cyanolyca* has very deep roots. Similarities in length of this region are also evident between some genera, such as scrub jays (*Aphelocoma*) and crested jays (*Cyanocitta*), and *Calocitta* and *Psilorhinus.* These patterns strengthen the hypothesis of relationships based solely on sequence differences (Fig. 5), despite the fact that phylogenetic analysis of the length-variable region itself did not yield results consistent with the rest of the CR (not shown).

The position of *Cyanocitta* is critical to scenarios of the evolution of cooperative breeding in NWJs. Our CR data never placed *Cyanocitta* in a basal position to all other NWJs, although the reduced taxon set, total molecular tree implied that only *Cyanolyca* was more basal. From a parsimony perspective, therefore, the mtDNA data suggest that CB is a primitive trait to all NWJs that has been lost in the *Cyanocitta* clade and in western scrub jays, a pair-breeder with a genetic structure and demography quite different from its sister species, the Florida scrub jay (Burt 1996; McDonald et al. 1999). This scenario would suggest that the phylogenetic propensity for CB in NWJs has been overcome multiple times. However, our maximum likehood analysis suggested, counterintutively, that the rate of loss of CB was almost three times less than the rate of gain. This result likely arose because the the value of α required to maintain CB in most of the species in the face of some losses of CB is predicted by the Discrete program to be high (Pagel 1999). In addition, the likelihood that the ancestor of NWJs was cooperative was predicted to be only 68%. We nonetheless believe that the origin of CB may in fact be even deeper than the divergence of New World and Old World jays as there have been reports of cooperative behavior in *Perisoreus* (Waite and Strickland 1997) as well as in *Corvus* (Richner 1990). Extensive re-analysis of this idea and judicious consideration of natural history data (Brown 1987) will be required in the future as we learn more about sociality in all species of NWJs.

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