Combined Nuclear and Mitochondrial DNA Sequences Resolve Generic Relationships within the Cracidae (Galliformes, Aves)

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Abstract.—The Cracidae is one of the most endangered and distinctive bird families in the Neotropics, yet the higher relationships among taxa remain uncertain. The molecular phylogeny of its 11 genera was inferred using 10,678 analyzable sites (5,412 from seven different mitochondrial segments and 5,266 sites from four nuclear genes). We performed combinability tests to check conflicts in phylogenetic signals of separate genes and genomes. Phylogenetic analysis showed that the unrooted tree of ((curassows, horned guan) (guans, chachalacas)) was favored by most data partitions and that different data partitions provided support for different parts of the tree. In particular, the concatenated mitochondrial DNA (mtDNA) genes resolved shallower nodes, whereas the combined nuclear sequences resolved the basal connections among the major clades of curassows, horned guan, chachalacas, and guans. Therefore, we decided that for the Cracidae all data should be combined for phylogenetic analysis. Maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses of this large data set produced similar trees. The MP tree indicated that guans are the sister group to (horned guan, (curassows, chachalacas)), whereas the ML and Bayesian analysis recovered a tree where the horned guan is a sister clade to curassows, and these two clades had the chachalacas as a sister group. Parametric bootstrapping showed that alternative trees previously proposed for the cracid genera are significantly less likely than our estimate of their relationships. A likelihood ratio test of the hypothesis of a molecular clock for cracid mtDNA sequences using the optimal ML topology did not reject rate constancy of substitutions through time. We estimated cracids to have originated between 64 and 90 million years ago (MYA), with a mean estimate of 76 MYA. Diversification of the genera occurred approximately 41–3 MYA, corresponding with periods of global climate change and other Earth history events that likely promoted divergences of higher level taxa. [Combined data; Cracidae; divergence time; mitochondrial DNA; molecular phylogeny; nuclear DNA.]

Advances in molecular biology techniques and the elaboration of more realistic evolutionary models for DNA sequences in the last decades have increased our knowledge of the mechanisms and patterns of nucleic acid and protein evolution and have led to an improvement in methods used to recover the phylogenetic history of organisms. Phylogenetic reconstruction using larger DNA data sets produce more reliable results than does reconstruction based on smaller data sets (Cao et al., 1994; Cummings et al., 1995; Russo et al., 1996; Nei et al., 1998). Moreover, the use of multiple genes for phylogenetic inference preferably should employ independent genes or genes regions, such as mitochondrial and nuclear genes or nuclear genes located in different chromosomes (e.g., Doyle, 1992). This approach helps avoid problems related to hybridization, gene transfer, and lineage sorting (Maddison, 1997).

The use of sequences from multiple genes raises the much debated issue of whether they should be analyzed separately (Miyamoto and Fitch, 1995) or combined (Kluge, 1989) in phylogenetic inference (see Huelsenbeck et al., 1996, for a review). In a partitioning approach, congruence between topologies obtained by independent data sets suggests that the gene tree reflects the species tree, providing a more confident estimate of phylogenetic relationships (Doyle, 1992; Prychitko and Moore, 1997; Johnson and Clayton, 2000). When topologies are incongruent, some of the genes or gene regions might not be useful for phylogenetic inference because of differences in their evolutionary patterns, thus hindering the recovery of the evolutionary history (Penny and Hendy, 1986; Mason-Gamer and Kellogg, 1996; Cunningham, 1997; Maddison, 1997). Additionally, some data partitions might violate assumptions of phylogenetic methods such as stationarity of base composition among taxa (Cunningham, 1997). Partitioning of data sets also makes phylogenetic analysis more sensitive to sampling variation because of lack of independence of

characters in a data set (Huelsenbeck et al., 1996).

In a combinability approach, estimates of phylogenetic relationships should be more robust as more data are included, especially if data sets are homogeneous regarding their evolutionary history (Huelsenbeck et al., 1996). Different data sets can be useful for resolving phylogenetic relationships at different levels (e.g., deeper versus more recent branches), often improving tree resolution and support for internal nodes when analyzed together (e.g., Olmstead and Sweere, 1994; Weller et al., 1994; Giannasi et al., 2001; but see Johnson and Clayton, 2000). However, heterogeneity in sequence data sets can sometimes impede phylogenetic inference, and the causes of incongruence between different data sets may pass undetected (Bull et al., 1993; Miyamoto and Fitch, 1995). Even evolutionary patterns typical of individual genes or genomes can be obscured because gene trees and species trees can be decoupled from a common history (Neigel and Avise, 1986; Pamilo and Nei, 1988; Avise, 1991; Doyle, 1992; Page, 2000).

A third approach to this problem is the conditional combination of sequence data sets (Bull et al., 1993; de Queiroz, 1993; Rodrigo et al., 1993). This approach can be used to distinguish between data partitions that provide incongruent and congruent estimates of a phylogeny. When congruent data partitions are detected, their combination is suggested in a combined data approach. Congruence of partitions can be assessed statistically by bootstrapping (de Queiroz, 1993), incongruence length difference tests (Farris et al., 1994), or likelihood ratio tests (LRTs; Huelsenbeck and Bull, 1996). The difficulties of the conditional approach involve how to choose among all possible partitions and how to deal with different data partitions that result in significantly different tree estimates. Most importantly, it is hard to determine how frequently putative partition heterogeneity occurs because of the error associated with statistical tests that indicate that data should not be combined when they actually present no conflict (Huelsenbeck et al., 1996).

In this study, we investigated the possibility of combining independent sets of DNA sequences to estimate the relationships among the 11 genera in the Cracidae. Our data set includes four nuclear genes comprising possibly three independent data sets (RAG-1/RAG-2, c-mos, and the seventh intron of β-fibrinogen) and seven different gene regions of mitochondrial DNA (12S ribosomal DNA [rDNA], CO1, CO2, CO3, cyt b, ND2/tRNA[Trp], ND5). We chose this galliform family because existing hypotheses of relationships among its genera have not been established previously with rigorous phylogenetic analysis or with complete sampling of genera. According to more recent phylogenetic analysis based on molecular data, Megapodiidae is the sister clade to other Galliformes (Groth and Barrowclough, 1999), and within them, Cracidae is a sister clade to Phasianidae, Odontiphoridae, and Numididae (Dimcheff et al., 2000). About 50 species and more than 60 subspecies are currently assigned to 11 genera (Vaurie, 1968) in three morphological subgroups: guans (*Aburria, Chamaepetes, Oreophasis, Penelope, Penelopina*, and *Pipile*) and chachalacas (*Ortalis*) of the subfamily Penelopinae and curassows (*Crax, Mitu, Nothocrax*, and *Pauxi*) of the subfamily Cracinae (Nardelli, 1993; del Hoyo et al., 1994). A large number of these species are listed in the *Endangered Species Red Data Book* (Collar et al., 1992; Brooks and Strahl, 2000), making this bird family one of the most endangered in the Neotropics. Thus, cracids can be considered keystone indicators for ecosystem preservation and management, and an important first step in understanding their evolutionary history is to establish a well-supported phylogeny of the extant genera.

MATERIAL AND METHODS
Taxa

We selected one species within each of the cracid genera to be representative of the genus (Table 1). One megapode, *Megapodius reinwardti* (Galliformes), and two screamers, *Anhima cornuta* and *Chauna torquata* (Anseriformes), were used as representative outgroups following Groth and Barrowclough (1999) and Dimcheff et al. (2000). DNA was extracted from blood by adding it to a solution of 0.1% SDS, 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, and 10 mg/ml proteinase K for 16–18 hr at 37°C. The DNA was purified using Tris-HCl saturated buffered phenol and chloroform/isoamyl solution.

TABLE 1. Cracid species used in this study, and geographic distribution of the genera they represent (Delacour and Amadon, 1973; Nardelli, 1993).

Group	Species	Historical distribution of the genus
Guans	*Aburria aburri*	Andes of Colombia, Ecuador, Peru, Venezuela
	Chamaepetes goudotti	Mountain forests of Central America, Colombia, Peru
	Penelope obscura	Mountain and lowland forests of South and Central America, Mexico
	Penelopina nigra	Wet mountain forests of Central America, Mexico
	Pipile jacutinga	Lowland forests of South America
Horned guan	*Oreophasis derbianus*	Mountain forests of Mexico and Guatemala
Chachalacas	*Ortalis canicollis*	Brushy areas of South, Central, and North America
Curassows	*Crax blumenbachii*	Mountain and rain forests of South and Central America, savanna in South America
	Mitu tuberosa	Amazon and Atlantic forests in South America
	Nothocrax urumutum	Forest around Upper and Middle Amazon River in South America
	Pauxi pauxi	Rain and cloud forests of Bolivia, Colombia, Peru, Venezuela

PCR and Sequencing

PCR amplifications were performed according to the protocol described by Hagelberg (1994). Amplified fragments were submitted to cycle sequencing, and sequences were obtained on a Li-Cor 4200 bidirectional automated DNA sequencer according to the manufacturer's suggested protocol. Mitochondrial regions sequenced for the cracid species listed in Table 1, and outgroups were 12S rDNA, CO1, CO2, CO3, cyt b, ND2/tRNA^{Trp}, and ND5. Nuclear genes sequenced were exons from RAG-1, RAG-2, and c-mos as well as the seventh intron of β-fibrinogen for the same taxa. Sequences are in GenBank under accession numbers AF165441–AF165512, AY140699–AY140792.

Sequence Alignments

Both DNA strands were checked for ambiguities using Sequencher 4.1.2 for Mac (GeneCodes Corp.). Edited sequences were aligned by eye in MacClade 4.0 (Maddison and Maddison, 2000). The final concatenated alignment contains 10,800 sites.

Phylogenetic Analysis

Prior to phylogenetic reconstruction, we performed tests of stationarity of base composition in TREEPUZZLE 5.0 (Strimmer and Von Haeseler, 1996) because compositional bias among species can interfere with tree topology (Foster and Hickey, 1999; Mooers and Holmes, 2000; Haddrath and Baker, 2001). Each gene was tested separately, and only variable sites were considered because inclusion of constant sites can hinder the de-tection of bias in base composition. Prior to phylogenetic analysis, sites containing gaps and overlapping regions of ND2 and tRNA^{Trp} were excluded from the alignment. Thus, the total number of analyzable sites was 10,678 sites (5,412 from seven different mitochondrial segments and 5,266 sites from four nuclear genes).

Tree searches to estimate phylogenetic relationships among the 11 genera of Cracidae were performed in PAUP 4.0 b10.0 (Swofford, 2001). The branch-and-bound algorithm was used to search for the maximum parsimony (MP) and maximum likelihood (ML) trees.

To ensure that an appropriate model of DNA evolution was used in ML analyses, we used the program MODELTEST 3.0 (Posada and Crandall, 1998) to test models incorporating parameters such as base frequency, rates of transition between purines and between pyrimidines, rates of transversions, proportion of invariable sites, and rate variation among sites. Rate variation among sites was performed under three different assumptions: (1) a proportion of sites, I, was assumed to be invariable, and the variables sites were assumed to evolve at the same rate; (2) all sites evolved according to a discrete gamma distribution, Γ; and (3) the same as (1) but the variable sites were allowed to evolve according to Γ. To speed up likelihood computation, the parameters previously obtained by MODELTEST 3.0 were used as user-defined parameters in PAUP 4.0 b10.0.

Bayesian analysis with Markov Chain Monte Carlo sampling was also carried out with MrBayes 2.01 for the combined data set and the nuclear and mitochondrial data partitions (Huelsenbeck and Ronquist, 2001). The analysis was run for 1 million

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generations, with one cold and three heated chains and a burn-in time determined by the time to convergence of the likelihood scores. One tree in every 1,000 trees was sampled to guard against autocorrelation. The model chosen was that used for the ML analysis.

Branch support for each data partition was given by bootstrapping (100 replicates) using branch-and-bound for MP and heuristic search for ML with random addition of sequences. This procedure was applied for all separate and combined analyses. In the Bayesian analysis for the combined, nuclear, and mitochondrial data sets the posterior probabilities of each node were computed across the sampled trees after burn-in.

Combinability Tests

To decide whether genes should be combined or partitioned in phylogenetic analysis, we performed LRTs (Huelsenbeck and Rannala, 1997) of the three unrooted trees that exist for guans, curassows, chachalacas (*Ortalis*), and the horned guan (*Oreophasis*) (Fig. 1). Tests were done separately for each mitochondrial and nuclear gene individually, for all mitochondrial data combined, for all nuclear genes combined, and for combined nuclear and mitochondrial DNA (mtDNA) sequences.

Parametric Bootstrapping for Comparisons of Competing Phylogenetic Trees

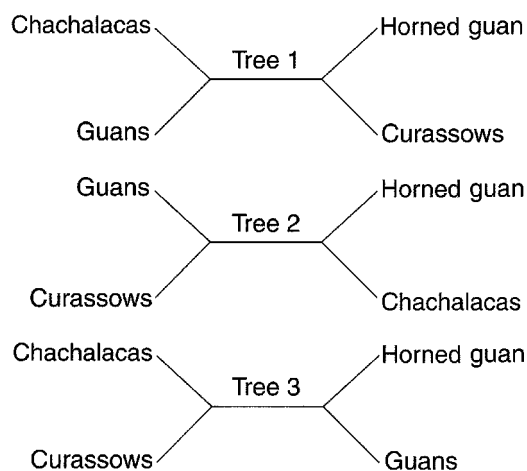To compare the topologies of the optimal MP and ML trees, we performed paramet-

FIGURE 1. All three possible unrooted trees for guans, curassows, the horned guan (*Oreophasis*), and chachalacas (*Ortalis*).

ric bootstrapping and a posteriori significance tests (SOWH test, Huelsenbeck and Bull, 1996; Swofford et al., 1996). The MP tree was used as a model tree for parameter estimation and to generate 100 replicate data sets in Seq-Gen 1.2.5 (Rambaut and Grassly, 1997) that had uniform base composition. Goldman et al. (2000) showed that this test has increased power in rejecting the null hypothesis and is more appropriate than the widely used nonparametric tests for comparisons of a posteriori hypotheses. Branch swapping was performed to ensure that the tree search did not get trapped in local optima. The differences between the log likelihoods of the MP and ML trees were compared with the distribution of the differences between each parametric replicate and the MP model tree. The same test was performed on classical hypotheses of phylogenetic relationships by Vaurie (1968) and Delacour and Amadon (1973) using their trees (Fig. 2) as the model tree for parameter estimation. These authors did not actually perform phylogenetic analysis using modern cladistic techniques, but instead the trees represent their thoughts about relationships of cracid genera. To render a tree representing the ideas of Delacour and Amadon consistent with current classification of the Cracidae into 11 genera, we split their *Crax* into *Crax*, *Mitu*, and *Pauxi* and their *Aburria* into *Aburria* and *Pipile*. Thus, zero branch lengths were assigned to the branches connecting these taxa in the tree used for parameter estimation to perform the SOWH test for Delacour and Amadon's hypothesis.

Molecular Dating of Divergence Times

The ML tree obtained using the best-fit model was forced to be clocklike, and its likelihood was compared with that of the nonclock ML tree through an LRT assuming a χ^2 distribution with number of taxa minus 2 df. We then used the method described by Haubold and Wiehe (2001) to estimate divergence within Cracidae and to obtain the confidence intervals for these estimates. In this method, it is assumed that the number of nucleotide substitutions and the substitution rate for a particular pair of taxa may be different from those for any other pair, and they are modeled according to a gamma distribution. A pair of sequences with a known time of divergence (the calibration point) are
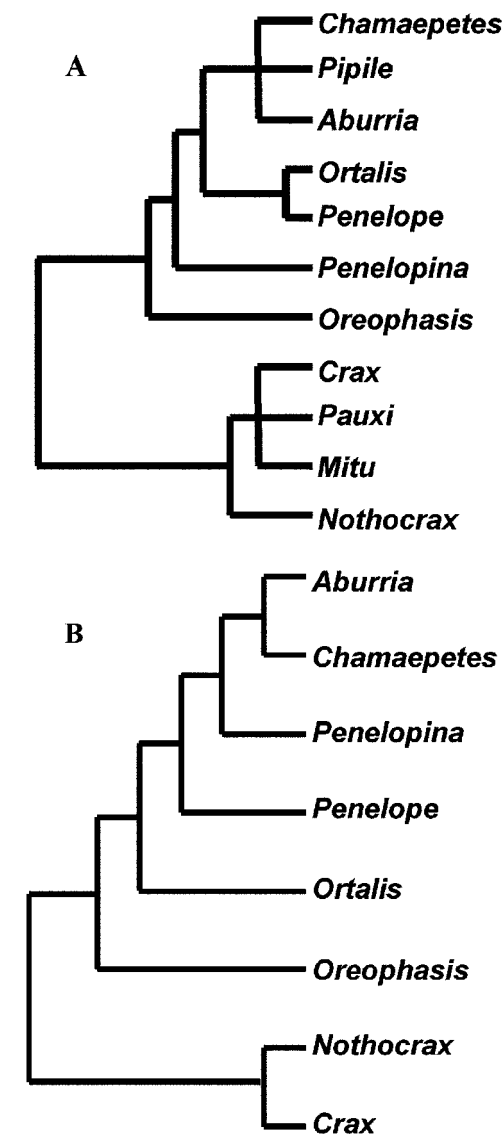
FIGURE 2. Inferred phylogenetic relationships of Cracidae of Vaurie (1968; A) and Delacour and Amadon (1973; B). In their classification, Delacour and Amadon merged *Aburria* and *Pipile* into *Aburria*, and *Crax* included *Crax*, *Mitu*, and *Pauxi*.

RESULTS

Sequences and Combinability Tests

Mitochondrial genes are known to have nuclear homologues in birds (Quinn, 1997), but we are confident that our sequences are of mtDNA origin because (1) we obtained single PCR products and clean single sequences, (2) the cracid sequences were similar to other avian mitochondrial sequences deposited in GenBank, and (3) predictions of secondary structure for ribosomal genes (not shown) and the reading frame of protein-coding genes were similar to those of other birds. Amplification of nuclear genes also resulted in a single PCR band, and the sequences were highly similar to corresponding avian sequences deposited in GenBank, and no stop or start codons were detected within the nuclear protein-coding sequences.

Within cracids, transitions (TS) and tranversions (TV) accumulated linearly with uncorrected distances, indicating that the sequences obtained in this study are not saturated for either mitochondrial or nuclear genes. Transitions at third codon positions showed saturation in comparison among outgroups and between cracids and outgroups for cyt b. The mean base composition in the two cracid genome partitions revealed the expected bias toward lower G content and higher C content in mtDNA relative to nuclear DNA sequences (Fig. 3). Among cracids, the mean TS/TV ratio was 2.9 for concatenated nuclear genes sequences compared with 7.7 for the mitochondrial sequences and 6.4 for both genomes combined.

Tests for stationarity of base composition among taxa using only variable sites for each nuclear and mitochondrial gene revealed that cracids did not differ significantly in their base content ($P > 0.05$); no ingroup taxa

used as a reference in calculating the age of nodes in the tree. The split of Anseriformes and Galliformes at 85 million years ago (MYA) was used as calibration point (as estimated by Haddrath and Baker, 2001) using complete mitochondrial genomes of birds; this date is close to the date (90 MYA) recently estimated for the same node by van Tuinen and Hedges (2001) based on nuclear genes.

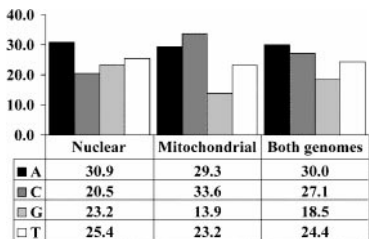|	Nuclear	Mitochondrial	Both genomes
■ A	30.9	29.3	30.0
▦ C	20.5	33.6	27.1
▨ G	23.2	13.9	18.5
☐ T	25.4	23.2	24.4

FIGURE 3. Mean base composition observed for nuclear, mitochondrial, and the combined sequence data set for Cracidae genera.

TABLE 2. Likelihood ratio tests between the ML tree and the alternative trees of Figure 1 for each data partition.

Partition	Tree 1	Tree 2	Tree 3
Both genomes	ML	15.98*	7.80
Nuclear	ML	7.58	24.15*
β-fibrinogen	1.95	ML	1.89
RAG-1	ML	7.07	7.33
RAG-2	ML	1.77	3.23
c-mos	ML	5.31	6.72
Mitochondrial	ML	7.16	2.59
12S rDNA	ML	2.95	2.73
CO1	1.16	1.16	ML
CO2	ML	4.53	0.82
CO3	1.25	1.13	ML
Cyt b	0.43	0.43	ML
ND2tRNA	ML	3.55	3.29
ND5	ML	3.40	1.87

$^*P < 0.05$.

failed the test. However, outgroup taxa failed for some data partions: *Megapodius* for RAG-2 ($P = 0.041$) and CO2 ($P = 0.008$); *Anhima* for RAG-1 ($P = 0.023$), 12S rDNA ($P = 0.034$), CO2 ($P = 0.046$), and ND2 ($P = 0.005$); and *Chauna* for cyt b ($P = 0.013$). However preliminary phylogenetic analyses including and excluding these genes resulted in similar tree topologies, and no instances of long-branch lengths that could result in long branch attraction during tree reconstruction were seen for these birds. Therefore, there is no reason to exclude any gene or outgroup taxa from subsequent analysis. Moreover, preliminary analysis using one to four different noncracid birds as outgroups did not change ingroup topology (results not shown).

We performed an LRT of all three unrooted trees for guans, curassows, chachalacas, and the horned guan *Oreophasis* (Fig. 1; Table 2). Tree 1 was the most likely topology when most genes were analyzed separately, when nuclear genes or mitochondrial genes were combined, and when genes from both genomes were combined.

Although the loglikelihoods indicated that tree 2 was the best tree for β-fibrinogen intron 7 and tree 3 was best for CO1, CO3, and cyt b, the difference between these two trees and tree 1 was not significant. Mean uncorrected distances among the four main groups within the Cracidae were around 5.8–6.2% (Table 3), but they were about 13% divergent from outgroups. The general time-reversible (GTR)+I+Γ distances are also shown in Table 3, and they do not differ greatly from uncorrected distances.

Phylogenetic Analysis

All codon positions in the mitochondrial protein-coding genes were included in phylogenetic analysis, because the exclusion of third positions can result in loss of phylogenetic signal, especially for closely related taxa (Edwards et al., 1991; Weller et al., 1994; Hästad and Björklund, 1998; Björklund, 1999; Källersjö et al., 1999; Yoder and Yang, 2000). Analyses excluding third codon positions resulted in poorly resolved trees that had polytomies within curassows, and among curassows, guans, chachalacas, and the horned guan (not shown). Among the analyzable sites used for the reconstruction of phylogenetic trees, 7,851 were constant and 1,869 were parsimony informative. MP analysis of the mitochondrial and nuclear genomes concatenated recovered a tree (Fig. 4) with 5,106 steps (consistency index = 0.672; retention index = 0.620; rescaled consistency index = 0.416). This tree showed guans to be a sister clade to ((curassows, chachalacas) horned guan)).

Model selection for ML performed through the hierarchical LRT (Posada and Crandall, 1998) showed that the GTR model assuming a proportion of sites to be invariable (I = 0.488) and substitution rate variation among sites (Γ = 0.557) was the best fit to the combined nuclear and mitochondrial data set (Fig. 5). Base frequencies

TABLE 3. Mean (±SD) uncorrected distances (below diagonal) and GTR+I+Γ (above diagonal) for concatenated mitochondrial and nuclear DNA sequences for the four cracid groups defined in Figure 1 and the outgroups.

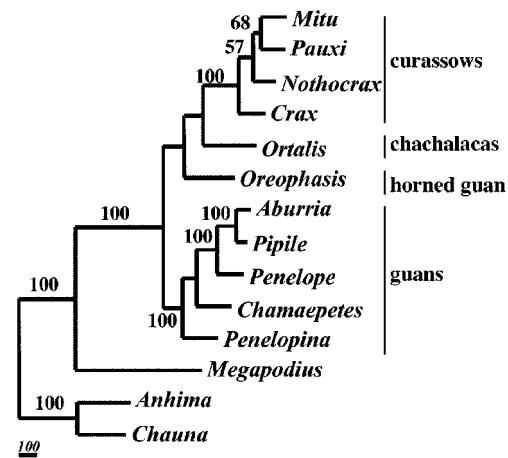|	Outgroups	Guans	Curassow	*Oreophasis*	Chachalacas
Outgroups		0.132 ± 0.0049	0.134 ± 0.0048	0.135 ± 0.0052	0.134 ± 0.0052
Guans	0.129 ± 0.0025		0.062 ± 0.0027	0.610 ± 0.0033	0.062 ± 0.0024
Curassows	0.132 ± 0.0022	0.061 ± 0.0017		0.061 ± 0.0030	0.058 ± 0.0025
Oreophasis	0.133 ± 0.0022	0.061 ± 0.0014	0.060 ± 0.0021		0.062 ± 0.0034
Chachalacas	0.130 ± 0.0021	0.060 ± 0.0016	0.058 ± 0.0018	0.062 ± 0.0021	

FIGURE 4. MP tree obtained from the combined sequence data. *Anhima, Chauna,* and *Megapodius* were used as outgroups. Numbers at nodes are bootstrap proportions obtained with 1,000 replicates. Branch lengths are proportional to number of changes.

were estimated to be 0.304, 0.286, 0.181, and 0.229 for A, C, G, and T, respectively. Analysis of these combined sequences with the GTR+I+Γ model in ML yielded a tree similar to the MP tree, but the horned guan was a sister clade to the curassows and the chachalacas were sister to this group. Also,
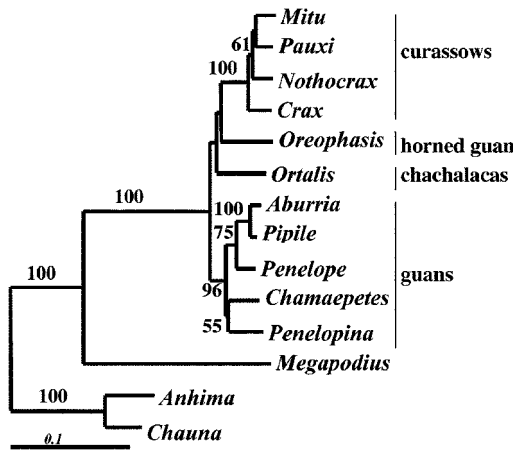

FIGURE 5. ML tree obtained using concatenated mitochondrial and nuclear genes under the GTR+I+Γ model of evolution. *Anhima, Chauna,* and *Megapodius* were used as outgroups. Numbers at nodes are bootstrap proportions for 100 replicates. Bayesian analysis resulted in a tree with the same topology and posterior probability of 100% for all nodes except that the horned guan was sister to the curassows with a posterior probability of 77% of the sampled trees and *Chamaepetes* and *Penelopina* formed a sister clade to the other guans with a posterior probability of 93%.

Penelopina and *Chamaepetes* were grouped in a sister clade to the other guans. Bayesian analysis resulted in a tree with the same topology as the ML tree.

To further investigate these differences between the MP and ML trees, we performed parametric bootstrapping for the combined nuclear and mitochondrial data set. The results of the parametric bootstrapping indicated that the difference between both trees (4.91 log-likelihood units) was significant at the 5% level (critical value = 0.590; Fig. 6). Moreover, the phylogenetic hypotheses of Vaurie (1968; Fig. 2) and Delacour and Amadon (1973; Fig. 2) were also rejected at the 5% level (Fig. 6).

Bootstrap values obtained for MP and ML analyses provided support for most parts of the tree. Nodes with support <50% include the relationship of the horned guan and the chachalacas to the curassows and the position of *Penelopina* and *Chamaepetes*. These taxa also differed in the MP and ML trees. However, Bayesian analysis provided better
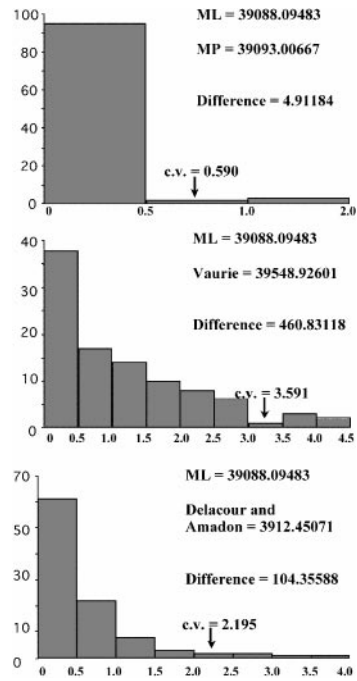

FIGURE 6. Distribution of the SOWH test statistic generated by parametric bootstrapping of 100 replicates of the combined sequence data set. Critical values (c.v.) that must be exceeded for a significant result at the 5% level are indicated above each histogram. Log likelihoods were obtained based on parameters estimated from the original data set.

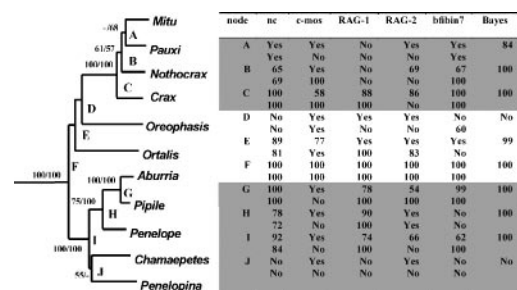| node	nc	c-mos	RAG-1	RAG-2	bfibin7	Bayes
A	Yes	Yes	No	Yes	Yes	84
	Yes	No	No	No	Yes	
B	65	Yes	No	69	67	100
	69	100	No	100	100	
C	100	58	88	86	100	100
	100	100	No	No	100	
D	No	Yes	Yes	Yes	No	No
	No	Yes	No	No	60	
E	89	77	Yes	Yes	No	99
	81	Yes	100	83	No	
F	100	100	100	100	100	100
	100	100	100	100	100	
G	100	Yes	78	73	99	100
	100	No	100	100	100	
H	78	Yes	90	Yes	No	100
	72	No	100	Yes	No	
I	92	Yes	74	66	62	100
	84	No	100	No	100	
J	No	Yes	No	Yes	No	No
	No	Yes	No	No	No	

FIGURE 7. Bootstrap proportions obtained by ML (top value) and MP (bottom value) for each node for the combined nuclear data set (nc) and for each nuclear gene. Bayes posterior probabilities of the Bayesian analysis performed for the concatenated nuclear genes. Upper and lower shaded blocks highlight the curassow and guan clades, respectively. "Yes" and "No" indicate presence or absence of the node in the optimum unrooted trees for the data partition compatible with the ML tree of Figure 5 but not supported by >50% of bootstrap replicates. The tree depicts the relationships shown in Figure 5, and the numbers are bootstrap proportions of the concatenated mitochondrial and nuclear sequences for the ML (left value) and MP (right value) analyses, respectively.

support values for the tree. Only two nodes has posterior probabilities <100%: The probability of *Chamaepetes* and *Penelopina* in a sister clade to other guans was 93%, and the placement of the horned guan as sister to curassows had a posterior probability of 77%.

When we analyzed each gene separately, neither nuclear nor mitochondrial genes alone could fully resolve the phylogenetic relationships of cracids (Figs. 7, 8). Furthermore, the genes provided support for different parts of the tree. For example, nodes C, D, E, and I in Figures 7 and 8 represent divergence between the main four groups within

TABLE 4. Estimates of divergence times (MYA) and confidence intervals (CI) of cracid genera. Nodes A–J correspond to those in Figures 7 and 8. The calibration point used was the split between Galliformes and Anseriformes at 85 MYA estimated by Haddrath and Baker (2001).

Taxa	Node	MYA	95% CI	Epochs
Aburria and *Pipile*	F	3.8	3.2–4.5	Early Pliocene
Mitu and *Pauxi*	A	8.1	6.6–9.8	Late Miocene
Nothocrax	B	9.4	7.9–11.0	Late Miocene
Crax	C	10.0	8.4–11.9	Late Miocene
Penelope	G	10.5	8.8–12.3	Late Miocene
Chamaepetes and *Penelopina*	I	18.6	15.7–21.9	Early Miocene
Within guans	H	19.1	15.9–22.6	Early Miocene
Ortalis	D	30.9	25.8–36.5	Early Oligocene
Oreophasis	E	31.1	26.6–36.1	Early Oligocene
Guans vs. other cracids	J	33.3	26.9–40.6	Early Oligocene

cracids, and they received higher bootstrap support from nuclear than from mitochondrial genes. The reverse was true for most of the shallower nodes, where combined mtDNA sequences provided much higher support than did nuclear genes.

Molecular Dating of Divergence Times

Cracid mtDNA sequences seem to evolve in a clocklike fashion ($\chi^2 = 21.026$, 12 df, $P > 0.05$ for the LRT). Using the calibration date of 85 MYA for the divergence of Anseriformes from Galliformes (Haddrath and Baker, 2001), the family Cracidae originated between 64 and 90 MYA ($\bar{x} = 76$ MYA). Modern genera were estimated to have differentiated between 40 and 3 MYA. The estimated dates and their confidence intervals are summarized in Table 4.

DISCUSSION
Combining Versus Partitioning of Genes

The use of combined DNA sequences from nuclear and mitochondrial genomes provided the best estimate of the phylogenetic relationships of the 11 genera of the Cracidae because these sequences complement each other in providing stronger support for nodes at various depths in the phylogeny. These longer sequences also helped reduce stochastic error in substitutions across sites, as evidenced by increased bootstrap support at key nodes in the tree. Combinability tests with LRTs indicated that the two genomic

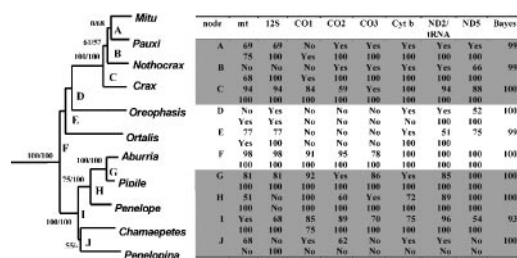| node	mt	12S	CO1	CO2	CO3	Cyt b	ND2/tRNA	ND5	Bayes
A	69	69	No	Yes	Yes	Yes	Yes	Yes	99
	75	100	Yes	100	100	100	100	100	
B	No	No	No	Yes	Yes	Yes	Yes	66	99
	68	100	Yes	100	100	100	100	100	
C	94	94	84	59	Yes	100	94	88	100
	100	100	Yes	100	100	100	100	100	
D	No	Yes	No	No	No	Yes	Yes	52	100
	Yes	100	No	No	No	100	100	100	
E	Yes	77	No	No	No	100	51	75	99
	Yes	100	No	No	No	100	100	100	
F	98	98	91	95	78	100	100	100	100
	100	100	100	100	100	100	100	100	
G	81	81	92	Yes	86	Yes	85	100	100
	100	100	100	100	100	100	100	100	
H	51	No	100	60	Yes	72	89	100	100
	100	No	100	100	100	100	100	100	
I	Yes	68	85	89	70	75	96	54	93
	100	100	75	100	100	100	100	100	
J	68	No	Yes	62	No	100	Yes	Yes	100
	No	100	No	No	No	No	No	No	

FIGURE 8. Bootstrap proportions obtained by ML (top value) and MP (bottom value) for each node for the combined mitochondrial data set (mt) and for each mitochondrial gene. Bayes posterior probabilities of the Bayesian analysis performed for the concatenated mitochondrial genes. See Figure 7 for additional details.

partitions were phylogenetically consistent across the cracid genera. The congruence between gene trees constructed with each of these large, independent partitions and with both combined increases confidence that the species tree was recovered in our analyses. Another reason to combine different gene partitions for cracid phylogeny is that none of the partitions recovered tree topologies that differed greatly at well-supported nodes in the combined analysis. All nodes supported at the 100% level in either ML and MP bootstrap analyses were also well supported in individual partitions.

Molecular phylogeneticists are increasingly employing nuclear DNA sequences to estimate relationships among taxa because of the independent estimates these genes provide of evolutionary history of the species in which they reside (e.g., Prychitko and Moore, 1997; Groth and Barrowclough, 1999; Armstrong et al., 2001; Roca et al., 2001). Part of the reason for this shift in choice of genes pertains to the nonindependence of mtDNA gene sequences because of linkage and the generally higher levels of homoplasy and substitutional bias in this quickly evolving organellar genome. However, judicious use of sequences from both genomes in conjunction with appropriate models of evolution can help resolve phylogenetic relationships that neither can resolve alone. This benefit of the combined approach is clearly illustrated in Figures 7 and 8; different genes provided support at nodes at different phylogenetic depths within the family Cracidae. Analysis obtained by MP, ML, and Bayesian analyses using sequences from both genomes combined resulted in similar trees. However, because MP does not account for a more elaborate model of DNA evolution suggested by the pattern of substitution in the sampled sequences, including correction for multiple hits at sites, the ML/Bayesian tree is taken here as the best estimate for the phylogenetic relationships among the 11 cracid genera.

Cracidae Systematics

The optimal ML/Bayesian tree (Fig. 5) disagrees with previous notions about relationships among cracid genera. Sclater and Salvin (1870) thought the Cracidae should be separated into three subfamilies based on postacetabular characters: Penelopinae (including guans and chachalacas), Oreophasi-

nae (including only the horned guan *Oreophasis*), and Cracinae (including curassows). Vaurie (1968) recognized the same three subdivisions but categorized them as tribes: Penelopini, Oreophasini, and Cracini, respectively. He argued that the horned guan was sufficiently distinct to deserve its own tribe and that it was a guan rather than a curassow and that the chachalacas were nested within guans (Fig. 2). More recent classifications have recognized only two subfamilies: the Cracinae containing the four curassow genera and the Penelopinae containing all other genera (Nardelli, 1993; del Hoyo et al., 1994). We suggest the separation of the Cracidae in two main groups: the first containing only the guan genera (*Aburria, Pipile, Penelope, Penelopina*, and *Chamaepetes*) in partial accordance with Sclater and Salvin's and Vaurie's classifications and the second containing the curassows (*Crax, Nothocrax, Pauxi*, and *Mitu*) and the chachalacas (*Ortalis*) and the horned guan (*Oreophasis*). Based also on the uncorrected mean distances among the four major cracid lineages (Table 3), each one of these lineages could deserve their own taxonomic rank within Cracidae.

Tempo and Mode of Differentiation Among the Cracid Genera

Although estimates of divergence time based on the fossil record, biogeographic events, or other molecular date estimates are common practice in molecular phylogenetic studies (e.g., García-Moreno and Silva, 1997; Knowlton and Weigt, 1998; Kumar and Hedges, 1998; van Tuinen et al., 1998; Arnaiz-Villena et al., 1999; Haddrath and Baker, 2001; Paton et al., 2002), caution is required in assuming these estimates as absolute dates. The quite large 95% confidence intervals associated with these estimates of dates of divergence testify to this uncertainty. Although we estimated that the cracid lineage originated approximately 76 MYA when they split from their sister group, the megapodes (Cracraft, 1981; Dimcheff et al., 2000; Barrowclough and Groth, 2001), the 95% confidence intervals span the period from 64 to 90 MYA. Cooper and Penny (1997) suggested cracids originated well before the Cretaceous–Tertiary boundary and therefore must have survived this mass extinction event, but our data suggest that

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origin after this event cannot be discounted absolutely (although it is unlikely). Irrespective of these broad confidence limits, the Gondwanan distribution of megapodes in Australia and cracids in South America fits a vicariance scenario, because both continents separated from Antarctica within the last 45 million years (MY) (Smith et al., 1981; Flynn and Wyss, 1998; Cracraft, 2001; Li and Powell, 2001).

The crown group of modern cracids was estimated to have a common ancestry between 27 and 41 MYA (95% CI, $\bar{x} = 33$ MYA), with the split between the lineages leading to guans and the clade composed of the horned guan, chachalacas, and curassows. These dates are in agreement with the oldest fossils attributed to cracids, *Procrax brevipes* (Tordoff and MacDonald, 1957) and *Palaeonossax senectus* (Miller, 1944), from the Early and Late Oligocene, respectively. However, without a thorough phylogenetic analysis of extant and extinct forms, these fossils are not useful as a check on the reasonableness of our estimates. Furthermore, Crowe and Short (1992) argued that all pre-Miocene fossils belong to an extinct family and thus are part of the stem group. Our estimate for the common ancestry of modern genera implies an interval of at least 23 MY (based on the 95% CIs) and possibly about 45 MY (based on the means) after the origin of the family Cracidae. A similar pattern of an evolutionary gap is also present in other Neotropical birds, e.g., hummingbirds as suggested by DNA–DNA hybridization data (Bleiweiss, 1998) and parrots as estimated by MTDNA sequences (Miyaki et al., 1998). This time interval corresponds to significant changes in faunal composition during the Paleocene and to global warming during the Eocene, resulting in mean temperatures 15–20°C higher than today at low and middle latitudes (Koch et al., 1992; MacFadden, 2000; Zachos et al., 2001). These events might have changed Earth conditions such that many older forms were replaced by new radiations. However, without a better fossil record for Neotropical birds it will be difficult to determine whether they were less diverse before the Miocene, whether Miocene lineages replaced older lineages, or whether assumptions are correct (Bleiweiss, 1998).

The warm climate of the Eocene followed by a significant cooling in the Miocene approximately 39–32 MYA are known to have influenced the extinction, diversification, and geographic range shifts of diverse groups such as plants, marine invertebrates, amphibians, reptiles, and land mammals (MacFadden, 2000). This was also the approximate time when the crown group of cracids originated and diversified into guans, the horned guan, chachalacas, and curassows.

The diversification within the four main lineages of cracids might be attributed to ecological and biogeographic causes. For example, the origin of the chachalaca lineage that today is found in forests and in brushy areas of the Americas might have been favored by the formation of arid, colder open-savanna-like habitats during the Oligocene (Petri and Fúlfaro, 1983; MacFadden, 2000). *Chamaepetes*, *Penelopina*, and the lineage leading to *Penelope, Pipile*, and *Aburria* diverged from each other between 16 and 23 MYA ($\bar{x} = $ 19–18 MYA), at a time when South America was segmented by marine transgression and the Andean orogeny (Petri and Fúlfaro, 1983; Lundberg et al., 1998). Similarly, the diversification of curassows between 12 and 6 MYA (95% CI) coincides roughly with the formation of the Eastern and Western Andean Cordillera during the late Miocene and Pliocene, and with changes in river basins due to Andean orogeny. Until a more detailed phylogenetic study with much more extensive sampling of cracid species is completed and speciation events are dated with more precision, the role of vicariance must remain an intriguing but untested possibility. Dispersal of the common ancestor of guans and chachalacas to Central and North America cannot be ruled out, because their divergence time is in agreement with the existence of a Costa Rica–Panama island arc (Meschede and Frisch, 1998).

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