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MOLECULAR PHYLOGENETICS OF THE *RAMPHASTOS* TOUCANS: IMPLICATIONS FOR THE EVOLUTION OF MORPHOLOGY, VOCALIZATIONS, AND COLORATION

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Abstract.—I reconstructed the phylogeny of 12 *Ramphastos* toucan taxa using mitochondrial DNA (mtDNA) sequences. This analysis identified two major groups, including a monophyletic smooth-billed yelping clade and a clade including most, but not all, the channel-keel-billed croakers. Within the *R. tucanus* and *R. vitellinus* groups, uncorrected mtDNA divergences are relatively low and mtDNA sequences from several subspecies are paraphyletic. One exception to low divergences within the *R. vitellinus* group is *R. v. ariel* from southeastern Brazil, which on average differs from all other *R. vitellinus* sampled by 2.9%. Character reconstructions on the phylogeny indicate that the ancestral *Ramphastos* was most likely a large-bodied channel-keel-billed croaker. Furthermore, an assessment of the patterns of bill shape, voice, and both plumage and bare-part coloration characters suggests that bill shape and voice have significant phylogenetic signal but that color characters do not. Sympatric *Ramphastos* taxa are not closely related in the phylogeny; therefore, character reconstructions indicate that the extreme similarity in coloration patterns between many sympatric *Ramphastos* pairs is most likely attributable to a combination of convergence or parallelism (homoplasy) and shared ancestral character states (symplesiomorphy). *Received 28 January 2004, accepted 5 April 2005.*

Key words: character reconstruction, coloration, phylogeny, *Ramphastos*, toucans.

Filogenética Molecular de los Tucanes del Género *Ramphastos*: Implicaciones para la Evolución de la Morfología, las Vocalizaciones y la Coloración

Resumen.—Reconstruí la filogenia de 12 taxa del género *Ramphastos* (Ramphastidae) usando secuencias de ADN mitocondrial (ADNmt). Este análisis identificó dos grupos principales, incluyendo un grupo monofilético compuesto por tucanes de picos lisos que emiten gañidos y un clado compuesto por la mayoría de los tucanes de picos acanalados que emiten graznidos. Dentro de los grupos de *R. tucanus* y *R. vitellinus* las distancias no corregidas en el ADNmt son relativamente bajas y las secuencias de varias subespecies son parafiléticas. Una excepción al patrón de divergencia limitada dentro del grupo de *R. vitellinus* es el caso de *R. v. ariel*, un taxón del sudeste de Brasil, cuyas secuencias difieren en promedio en un 2.9% con respecto a todos los demás *R. vitellinus* muestreados. Las reconstrucciones de la evolución de caracteres hechas con base en la filogenia indican que el *Ramphastos* ancestral probablemente presentaba tamaño corporal grande y pico acanalado y sus vocalizaciones eran graznidos. Además, una evaluación de los patrones de forma del pico, las vocalizaciones y la coloración del plumaje y de las partes desnudas

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sugiere que la forma del pico y las vocalizaciones son buenos indicadores de las relaciones filogenéticas, pero los caracteres de color no lo son. Los taxa simpátricos del género *Ramphastos* no están estrechamente relacionados de acuerdo a la filogenia. Por lo tanto, la marcada similitud en los patrones de coloración de muchos pares de especies simpátricas es probablemente atribuible a una combinación de convergencia o paralelismo (homoplasia) y a la retención de caracteres ancestrales compartidos (simplesiomorfía).

Ramphastos toucans are a particularly interesting group, because many sympatric pairs of *Ramphastos* taxa look strikingly similar to one another in plumage and bare-part coloration (Haffer 1974), yet they vary dramatically in body size, culmen shape, and vocalizations. The *Ramphastos* toucans are large-bodied, canopy-dwelling birds in the order Piciformes (woodpeckers and allies) and range from Mexico south to Argentina (Fig. 1). Most geographical forms of *Ramphastos* have conspicuous coloration differences and were therefore originally described as full species (Haffer 1974), with 11 to 15 diagnosable species recognized (de Germiny 1929; Peters 1948; Meyer de Schauensee 1966, 1970). However, Haffer (1974) and Short and Horne (2001, 2002) recognized only seven biological species of *Ramphastos*, which Haffer (1974) divided into two groups of apparently closely related species on the basis of bill shape and vocalizations. The channel-keel-billed *Ramphastos* are relatively small in body size (except *R. toco*) and have croaking vocalizations. The smooth-billed *Ramphastos* are relatively larger in body size and have yelping calls. In most lowland sites, two species of *Ramphastos*, usually one from the smaller-bodied channel-keel-billed group and one from

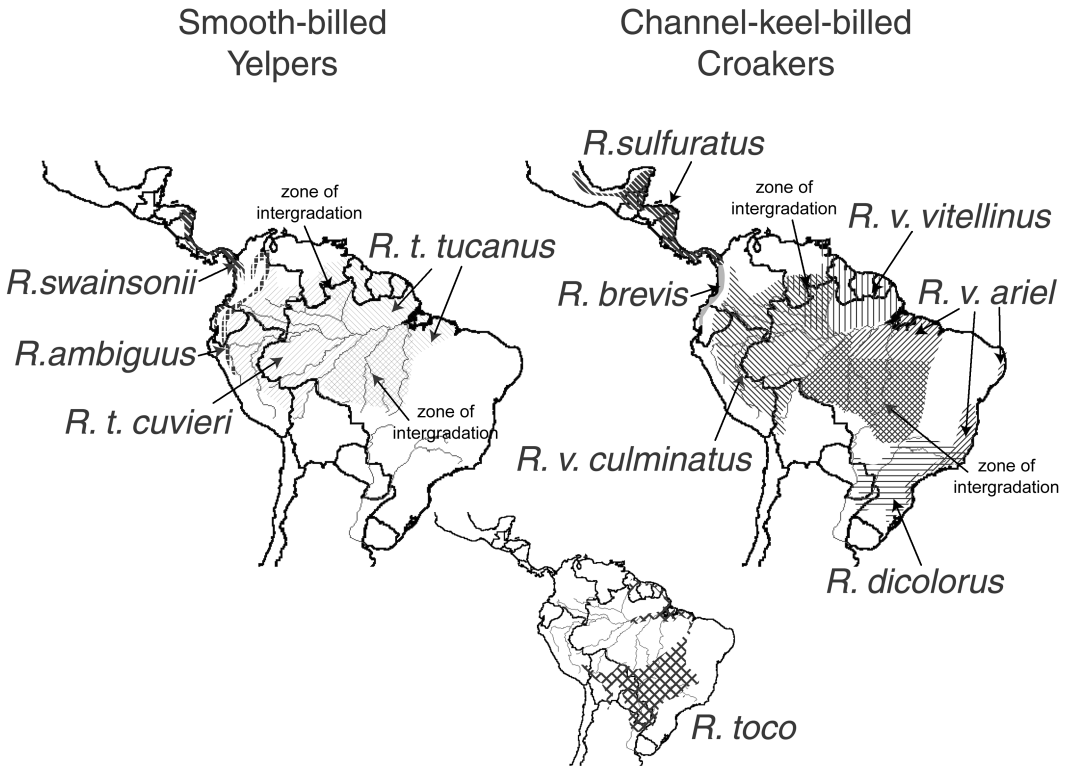

FIG. 1. Map showing the approximate distributions of *Ramphastos* toucans used in the study.

the larger-bodied smooth-billed group, are sympatric (Haffer 1974). At least four of these pairs of sympatric *Ramphastos* exhibit a striking pattern of similarity in coloration (Table 1; Haffer 1974, 1997a). For example, in western Amazonia, *R. tucanus cuvieri* and *R. vitellinus culminatus* share a yellow-ridged black bill, white throat, yellow uppertail coverts, and bluish orbital skin. Likewise, in the Choco, west of the Andes, *R. swainsonii* and *R. brevis* have a bicolored bill pattern, yellow throat, white uppertail coverts, and yellowish-green orbital skin. Within each pair, the component species look identical in all aspects of plumage and bare-part coloration and are the most extreme cases of similarity in *Ramphastos*. In Central America, *R. swainsonii* and *R. sulfuratus* are identical in plumage coloration and orbital skin coloration, but differ in bill coloration; a similar pattern is shown by *R. v. ariel* and *R. dicolorus* in southeastern Brazil. *Ramphastos v. ariel* and *R. dicolorus* are the only sympatric pair in which both species are members of the channel-keel-billed group. In this case, *R. v. ariel* and *R. dicolorus* differ in body size and in the quality of their croaking vocalization, but look similar in plumage and bare-part coloration (Haffer 1974). *Ramphastos v. ariel* and *R. dicolorus* are sympatric, however, only during the austral winter in southeastern Brazil (Haffer 1974).

Phylogenetic reconstructions of the evolution of culmen shape, vocalizations, and coloration patterns can shed considerable light on how these patterns evolved and can provide clues as to whether similarity is attributable to convergence (homoplasy) or retention of ancestral character states (symplesiomorphy). For *Ramphastos*, one of two conflicting character sets—either culmen shape and vocalizations, as suggested by Haffer (1974), or plumage and bare-part colors—is probably phylogenetically informative, whereas the other is convergent. However, without a robust phylogenetic hypothesis on which to reconstruct the evolutionary patterns of these characters, it is not possible to assess which sets of characters have phylogenetic signal and which are convergent.

I used mitochondrial DNA (mtDNA) sequences to estimate the phylogeny of the *Ramphastos* toucans. I used this phylogeny to assess patterns of similarity in voice, culmen shape, and coloration patterns and to examine whether they are convergent (homoplasious), shared ancestral (symplesiomorphic), or shared derived (synapomorphic) characters.

If bill shape and vocalization type have significant phylogenetic signal and if coloration patterns exhibit high levels of homoplasy, the results are consistent with Haffer (1974). Alternatively, if culmen shape and vocalizations lack significant phylogenetic signal (suggesting homoplasy) and if plumage and bare-part coloration have strong phylogenetic signal, then similarity in coloration of sympatric *Ramphastos* is caused by their close phylogenetic relationships. Insights gained from this historical phylogenetic approach can be used to test hypotheses of character evolution in this group and to provide a basis for future experimental work testing hypotheses of *Ramphastos* plumage evolution.

Methods

Samples, Polymerase Chain Reaction, and DNA Sequencing

I extracted DNA from frozen tissues of 22 *Ramphastos* and 5 outgroup taxa using the DNeasy extraction kit (Qiagen, Valencia, California). When possible, I included multiple individuals of each *Ramphastos* species or subspecies from different localities and have included all but two currently recognized subspecies (*R. v. citreolaemus* and *R. t. toco*) that are not considered intergrades (Short and Horne 2002). All tissue samples used here were vouchered with standard museum specimens (see Table 2 for voucher and locality data). For each specimen,

Table 1. Pairs of similar-looking sympatric *Ramphastos* toucans.

Small-bodied	Large-bodied	Location
R. vitellinus culminatus	*R. tucanus cuvieri*	Western Amazonia
R. brevis	*R. swainsonii*	Choco (west of Andes)
R. sulfuratus	*R. swainsonii*	Central America
R. dicolorus	*R. v. ariel*	Southeastern Brazil

TABLE 2. Specimens used in the study.

Species	Common name	Locality	Source [a]	ID number
		Ingroup		
Ramphastos tucanus tucanus	Red-billed Toucan	Guyana	KU	B1356
R. t. tucanus	Red-billed Toucan	Pará, Brazil	LSUMNS	B35550
R. t. cuvieri	Cuvier's Toucan	Loreto, Peru	LSUMNS	B27691
R. t. cuvieri	Cuvier's Toucan	Pando, Bolivia	LSUMNS	B9392
R. swainsonii	Chestnut-mandibled Toucan	Darién, Panama	LSUMNS	B2309
R. swainsonii	Chestnut-mandibled Toucan	Esmeraldas, Ecuador	LSUMNS	B11712
R. ambiguus	Black-mandibled Toucan	Zamora-Chinchipe, Ecuador	ANSP	4465
R. sulfuratus sulfuratus	Keel-billed Toucan	Campeche, Mexico	KU	B2007
R. s. brevicarinatus	Keel-billed Toucan	Colón, Panama	LSUMNS	B28577
R. dicolorus	Red-breasted Toucan	Caazapá, Paraguay	KU	B282
R. toco	Toco Toucan	Santa Cruz, Bolivia	LSUMNS	B1477
R. toco	Toco Toucan	Captive	LSUMNS	B10925
R. brevis	Choco Toucan	Pichincha, Ecuador	LSUMNS	B12175
R. brevis	Choco Toucan	Pichincha, Ecuador	LSUMNS	B34977
R. vitellinus vitellinus	Channel-billed Toucan	Guyana	KU	B1237
R. v. vitellinus	Channel-billed Toucan	Pará, Brazil	LSUMNS	B35638
R. v. culminatus	Yellow-ridged Toucan	Loreto, Peru	LSUMNS	B2860
R. v. culminatus	Yellow-ridged Toucan	La Paz, Bolivia	LSUMNS	B924
R. v. culminatus	Yellow-ridged Toucan	Loreto, Peru	LSUMNS	B7192
R. v. ariel	Ariel Toucan	São Paulo, Brazil	LSUMNS	B35555
R. v. ariel [b]	Ariel Toucan	Pará, Brazil	LSUMNS	B35586
R. v. ariel	Ariel Toucan	Pará, Brazil	LSUMNS	B35667
		Outgroups		
Andigena cucullata	Hooded Mountain Toucan	La Paz, Bolivia	LSUMNS	B1273
Aulacorhynchus prasinus	Emerald Toucanet	Darién, Panama	LSUMNS	B1373
Selenidera reinwardtii	Golden-collared Toucanet	Loreto, Peru	LSUMNS	B27756
Pteroglossus inscriptus	Lettered Araçari	Pando, Bolivia	LSUMNS	B8819
Baillonius bailloni	Saffron Toucanet	Caazapá, Paraguay	LSUMNS	B25891

[a] Tissue sources: KU = University of Kansas Museum of Natural History, LSUMNS = Louisiana State University Museum of Natural Science, ANSP = Academy of Natural Sciences of Philadelphia.

[b] Morphology of this specimen is consistent with *R. v. ariel*, except that it has greenish orbital skin coloration, which might indicate intergradation with *R. v. culminatus*.

I sequenced 2,468 base pairs (bp) from three mitochondrial genes: cytochrome oxidase I (COI) (379 bp), cytochrome *b* (1,048 bp), and nicotinamide adenine dinucleotide dehydrogenase subunit 2 (ND2) (1,041 bp). To amplify COI, I used primers L6625 and H7005 (Table 3; Hafner et al. 1994) and the following thermal cycling profile: 94°C for 2 min, 35 cycles of 94°C for 30 s, 46°C for 30 s, 72°C for 30 s, followed by 72°C for 7 min. For cytochrome-*b* amplifications, I used the external primers L14841 (Kocher et al. 1989) and either H4a (Harshman 1996) or H16065 (Table 3; Helm-Bychowski and Cracraft 1993). For some specimens, I also amplified cytochrome *b* in two smaller fragments using combinations of the external primers and internal primers, including either TOUCCBH or BARBCBH (Moyle 2004) and TOUCCBL or BARBCBL (Table 3; Moyle 2004). The following thermal cycling profile was used for cytochrome-*b* amplifications: 30 cycles of 90°C for 40 s, 50°C for 40 s, 72°C for 40 s, followed by 72°C for 5 min. I amplified ND2 using the external primers L5215 (Hackett 1996) and H6313 (Table 3; Sorenson et al. 1999). As with cytochrome *b*, for some specimens I amplified two smaller fragments of ND2 using combinations of the external primers with two internal primers, H5776TOUC and L5758TOUC (Table 3). For ND2 amplifications, I used the following thermal cycling profile: 94°C for 10 min followed by 35 cycles of 94°C for 40 s, 50°C for 40 s, 72°C for 40 s, followed by 72°C for 5 min.

Polymerase chain reaction (PCR) products were verified on a 1% agarose gel and purified using a Qiaquick PCR purification kit (Qiagen). I used the ABI Big Dye kit (version 2; Applied Biosystems, Foster City, California) and ~75 ng of purified PCR product to perform cycle sequencing reactions. Unincorporated dyes were removed from these sequencing reaction products using Centrisep columns (Princeton Separations, Adelphia, New Jersey) repacked with Sephadex G-50, and these sequencing reaction products were run on an ABI 377 DNA automated sequencer (Applied Biosystems). I used SEQUENCHER (version 3.1, GeneCodes, Ann Arbor, Michigan) to reconcile double-stranded sequences and to align sequences for phylogenetic analyses. All sequences used in this study are deposited in GenBank (AY959799–AY959879).

Phylogenetic Analyses

I estimated the *Ramphastos* phylogeny using maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian analyses as implemented in PAUP* (version 4.0b10; Swofford 2001) and MRBAYES (version 3.0b4; Huelsenbeck and Ronquist 2001). Genetic distances were

Table 3. Primers used for PCR and sequencing samples in the study.

Gene	Primer	Sequence
Cytochrome *b*	L14841 [a]	5′-GCTTCCATCCAACATCTCAGCATGATG-3′
	TOUCCBH	5′-GAGAARRATGGGTGRAATGG-3′
	BARBCBH [b]	5′-GAGAAGTANGGGTGGAAKGG-3′
	TOUCCBL	5′-CTTCCTNCTNCCATTCCTAATYRCAGG-3′
	BARBCBL [b]	5′-CTTCCTCCTNCCATTYCTAATCRCAGG-3′
	H16065 [c]	5′-GGAGTCTTCAGTCTCTGGTTTACAAGAC-3′
	H4a [d]	5′-AAGTGGTAAGTCTTCAGTCTTTGGTTTACAAGACC-3′
COI	L6625 [e]	5′-CCGGATCCTTYTGRTTYTTYGGNCAYCC-3′
	H7005 [e]	5′-CCGGATCCACNACRTARTANGTRTCRTG-3′
ND2	L5215 [f]	5′-TATCGGGCCCATACCCCGAAAAT-3′
	H5776TOUC	5′-GGCTGARYAGGCMTCAACCARAC-3′
	L5758TOUC	5′-TGNGAGATRGAGGAGAARGC-3′
	H6313 [g]	5′-CTCTTATTTAAGGCTTTGAAGGC-3′

[a] From Kocher et al. (1989).
[b] From Moyle (2004).
[c] From Helm-Bychowski and Cracraft (1993).
[d] From Harshman (1996).
[e] From Hafner et al. (1994).
[f] From Hackett (1996).
[g] From Sorenson et al. (1999).

calculated using PAUP* (Swofford 2001). I used the partition homogeneity test (Farris et al. 1994, 1995) as implemented in PAUP* (Swofford 2001) to compare phylogenetic signal and test for incongruence between the COI, cytochrome-*b*, and ND2 data sets.

For MP analyses, all characters were unordered and equally weighted. Maximum-parsimony trees were built using a heuristic search with tree bisection–reconnection (TBR) branch-swapping and 100 random-addition replicates. I bootstrapped the MP data using 1,000 heuristic search replicates with TBR branch-swapping and 10 random additions per replicate (Felsenstein 1985). I also performed similar MP analyses on a combined data set, including both molecular and morphological characters.

MODELTEST (version 3.06; Posada and Crandall 1998), which implements the general procedure of Cunningham et al. (1998) and Huelsenbeck and Crandall (1997), was used to select the simplest model of sequence evolution and obtain model parameters for ML analyses. Model parameters obtained using MODELTEST (Posada and Crandall 1998) included empirical base frequencies, six rate-substitution parameters, invariant sites, and a gamma distribution shape parameter. To evaluate the support for likelihood tree branches, I used 100 bootstrap replicates with TBR branch-swapping and one random addition per replicate.

For Bayesian analyses, I used a mixed model (GTR + I + G), with nine data partitions, consisting of the three codon positions for COI, cytochrome *b*, and ND2. This approach accounts for potential differences in evolutionary model parameters between the nine data partitions. I did not define the model parameter values *a priori*; instead, I estimated them as part of the analysis. All the model parameters (except topology and branch lengths) were set as unlinked between partitions. I ran Bayesian analyses for 4.0×10^6 generations with four incrementally heated Markov chains and the default heating values, and initiated the analyses with random starting trees. Trees were sampled from the Markov chains every 1,000 generations, and the log-likelihood scores for all these sampled trees were plotted against generation time to determine when log-likelihood values reached a stable equilibrium (Huelsenbeck and Ronquist 2001). I discarded all trees sampled prior to this equilibrium point as "burn-in" (Leaché and Reeder 2002).

Character Reconstruction

For each terminal taxon in the phylogeny, I examined museum specimens at the Louisiana State University Museum of Natural Science and used Novaes (1949), Van Tyne (1955), Haffer (1974), and Short and Horne (2001) as references to code body regions with variable coloration as characters (Table 4). All characters were scored as unordered and either binary or multistate. *Ramphastos* plumage is mostly black with a few distinct white or carotenoid-colored patches. In carotenoid-colored patches, they express a continuous range of yellows, oranges, and reds. Therefore, I followed the methodology of Omland and Lanyon (2000) and scored only the presence and absence of carotenoid coloration for these variable plumage patches. Bill shape, vocalization, and body size characters were taken from Novaes (1949) and Haffer (1974). I scored eight characters known to be variable among *Ramphastos* toucan species (Novaes 1949, Van Tyne 1955, Haffer 1974; Table 4).

For these eight characters, I used MACLADE (version 3.07; Maddison and Maddison 1992) to reconstruct patterns of character evolution and to assess whether characters have significant phylogenetic signal. I calculated the consistency index (CI) and retention index (RI) for all characters overall (the ensemble CI and RI of Maddison and Maddison [1992]) as well as for each individual character mapped onto the mtDNA *Ramphastos* phylogeny. Phylogenetic signal or inertia of these characters was assessed using Maddison and Slatkin's (1991) randomization procedure. For each character, I randomized the character states 1,000× on the *Ramphastos* phylogeny and compared the reconstructed number of character-state changes to the random distribution of character-state changes. For character reconstructions and tests of phylogenetic signal, the mtDNA *Ramphastos* phylogeny was pruned to a topology including only one individual per species or subspecies. This pruning prevented multiple sampling, which would bias the test toward rejecting the null hypothesis. Characters were reconstructed using both acctran and deltran optimization. For each of the four similar-looking pairs of *Ramphastos* and for each character lacking phylogenetic signal in the Maddison and Slatkin (1991) test, I tabulated the number of homoplasies and symplesiomorphies. This

Table 4. Matrix of *Ramphastos* character states.[a]

Taxon	Character number							
	1	2	3	4	5	6	7	8
Ramphastos vitellinus ariel (Amazonia)	0	0	0	1	1	0	0	0
R. v. culminatus	1	0	1	1	0	0	0	0
R. v. vitellinus	0	0	1	1	1	0	0	0
R. v. ariel (southeastern Brazil)	0	0	0	1	1	0	0	0
R. brevis	0	1	2	0	0	0	0	0
R. dicolorus	0	0	0	2	1	0	0	0
R. tucanus cuvieri	1	0	1	1	0	1	1	1
R. t. tucanus	1	0	1	1	0	1	1	1
R. swainsonii	0	1	2	0	0	1	1	1
R. ambiguus	0	1	1	0	0	1	1	1
R. sulfuratus	0	1	2	2	0	0	0	0
R. toco	1	1	3	2	0	0	0	1

[a] Key for characters and character states: (1) carotenoid throat coloration: 0 = present, 1 = absent; (2) carotenoid uppertail-covert coloration: 0 = present, 1 = absent; (3) orbital skin coloration: 0 = red, 1 = light blue, 2 = green, 3 = yellow; (4) bill pattern: 0 = bicolored, 1 = colored base, often with a yellow ridge, 2 = other complex color patterns; (5) red breast band: 0 = narrow, 1 = wide; (6) shape of culmen cross-section: 0 = channel- or keel-shaped, 1 = smoothly rounded; (7) vocalization type: 0 = croaking, 1 = whistled yelping; and (8) body size: 0 = small mean weight (<550 g), 1 = large mean weight (>550 g).

was done using both the acctran and deltran character reconstructions to assess whether overall similarity among sympatric *Ramphastos* pairs is attributable to homoplasy or retention of ancestral character states. Homoplasy could be caused by convergence or parallelism.

Results

Sequence Attributes

The aligned matrix of 2,468 bp of mtDNA sequence for 27 taxa (5 outgroup, 22 ingroup) provided a total of 853 variable characters, of which 636 were potentially parsimony informative. No *Ramphastos* had identical sequences when compared across all three genes. Therefore, all individuals were included in phylogenetic tree reconstructions. Among ingroup taxa, uncorrected sequence divergence ranged from 0.0% to 10.0% for all genes, from 0.0% to 9.0% for COI, from 0.1% to 10.9% for cytochrome *b*, and from 0.0% to 10.5 for ND2. Within species of *Ramphastos*, uncorrected sequence divergences ranged from 0.0% to 3.0% for all genes, from 0.0% to 2.6% for COI, from 0.1% to 3.4% for cytochrome *b*, and from 0.0% to 2.9% for ND2. These within-species uncorrected divergence values are elevated by relatively high pairwise comparisons among samples from the *R. vitellinus* group. Uncorrected divergences between *R. v. ariel* from southeastern Brazil and all other *R. vitellinus* samples average 2.9% for all genes, 2.5% for COI, 3.2% for cytochrome *b*, and 2.8% for ND2. Excluding the southeastern Brazilian *R. v. ariel*, within-species uncorrected divergences for *Ramphastos* are lower, ranging from 0.0% to 0.8% for all genes, from 0.0% to 0.8% for COI, from 0.1% to 1.3% for cytochrome *b*, and from 0.0% to 0.8% for ND2.

Plots of pairwise comparisons of uncorrected sequence divergence between gene regions indicate that COI has a slower rate of divergence than ND2 and cytochrome *b* (Fig 2). Rates of divergence for ND2 and cytochrome *b* are similar; however, at higher divergences, cytochrome *b* appears to saturate earlier than ND2 (Fig. 2). The partition homogeneity test between COI, cytochrome *b*, and ND2 indicated that there was not significant conflict among these data partitions ($P = 0.23$). Therefore, I combined COI, cytochrome-*b*, and ND2 data sets for all phylogenetic analyses.

Phylogenetic Analyses

Maximum parsimony analysis produced four most-parsimonious trees (tree length [TL] = 1668, CI = 0.61, RI = 0.78), and a consensus of 1,000 parsimony bootstrap replicates strongly supported the monophyly of the smooth-billed yelping *Ramphastos* (Fig. 3). The channel-keel-billed

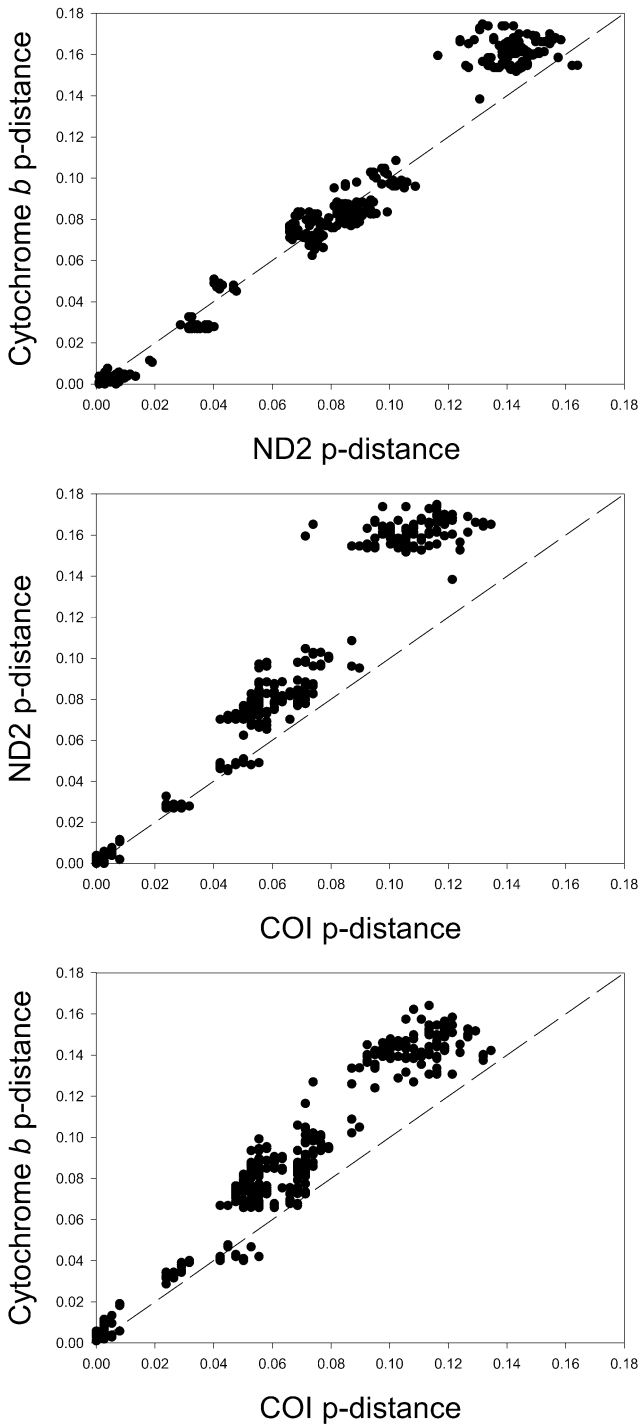

FIG. 2. Comparison of pairwise uncorrected divergences (*p*-distance) among mtDNA gene regions. Plots include ingroup and outgroup taxa. A dotted line of equal rates (slope of 1) is shown for comparison.

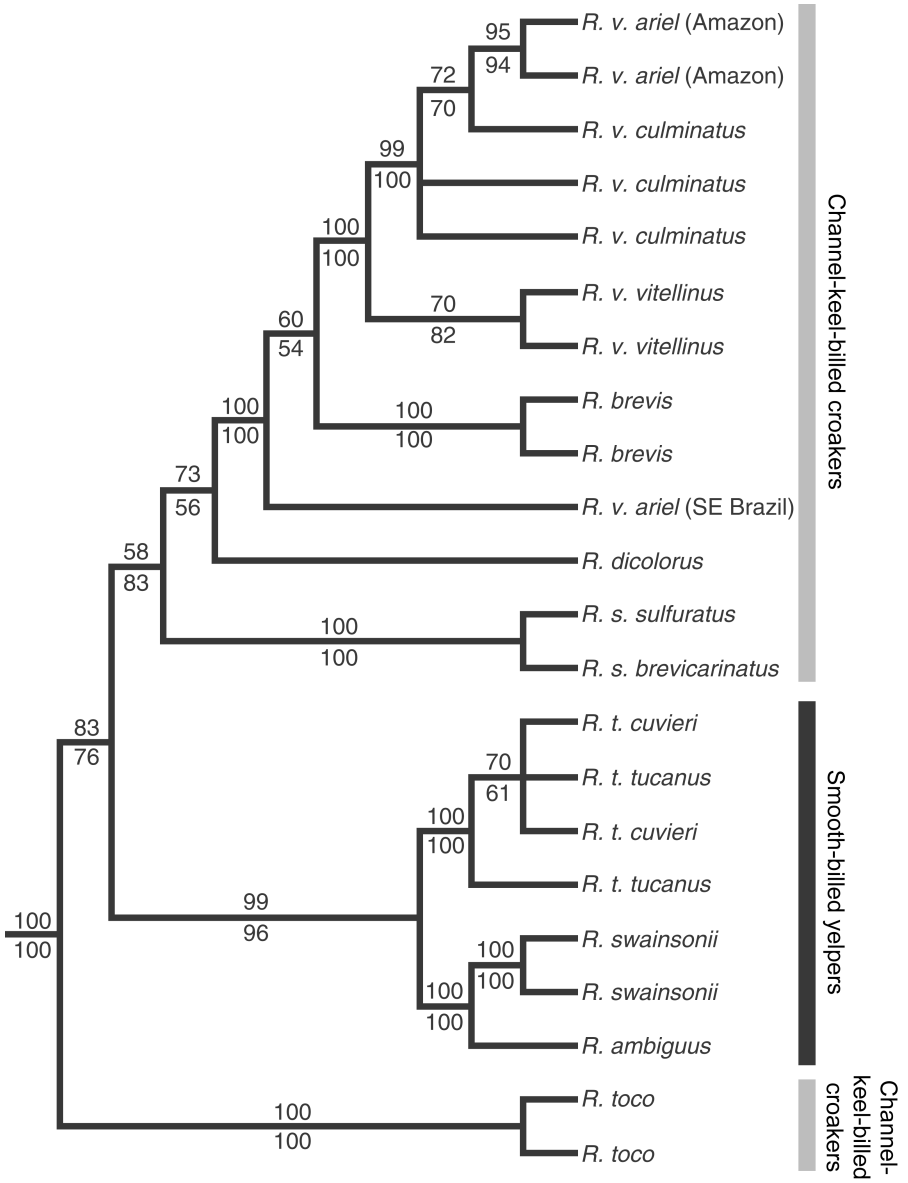

FIG. 3. The 50% majority-rule consensus tree summarizing the results of 1,000 MP bootstrap replicates and 100 ML bootstrap replicates from the combined mtDNA data set. Numbers above branches indicate percentage of MP bootstrap replicates in which the node was recovered. Numbers below branches indicate percentage of ML bootstrap replicates in which the node was recovered. Gray bars identify channel-keel-billed croakers and the black bar identifies smooth-billed yelpers.

croaking *Ramphastos* are not monophyletic, because the analysis places *R. toco* (a channel-keel-billed croaker) basal to all other *Ramphastos*. All but two resolved nodes in the consensus tree (Fig. 3) are supported by ≥70% of bootstrap replicates.

In the four most-parsimonious mtDNA gene trees, *R. v. ariel* is paraphyletic, with *R. brevis* sister to the *R. vitellinus* group to the exclusion of *R. ariel* from southeastern Brazil. This placement of *R. brevis* and *R. v. ariel* (from southeastern Brazil) is not strongly supported by bootstrapping,

but mtDNA paraphyly of *R. v. ariel* is strongly supported. The rest of the *R. vitellinus* group including Amazonian *R. v. ariel* forms a strongly supported monophyletic group. Bootstrapping also indicates reasonably strong support for the sister relationship between the two Amazonian *R. v. ariel* and one *R. v. culminatus*, to the exclusion of two other *R. v. culminatus*. Moderate bootstrap support (70%) for the clade including two *R. t. cuvieri* and one *R. t. tucanus* indicates that mtDNA sequences from the subspecies within the *R. tucanus* group may not be reciprocally monophyletic. The combined MP analysis of molecular and morphological characters is nearly identical to the molecular MP analysis except with respect to *R. v. culminatus*, which forms a weakly supported monophyletic group in the combined tree.

The topology of the ML tree (–lnL = 10973.58337; Fig. 4) is identical to one of the four most-parsimonious trees, and the ML bootstrap consensus tree is identical to the MP bootstrap consensus (Fig. 3). Levels of ML and MP bootstrap support are only minimally different. All but three resolved nodes in the bootstrap consensus tree (Fig. 3) were strongly supported by ≥70% of ML bootstrap replicates, whereas MP bootstrap values were <70% for only two resolved nodes.

All but one node in the Bayesian analysis is supported by ≥95% posterior probability (Fig. 4). Bayesian analysis produced a topology that was nearly identical to the ML tree and one of the four MP trees. The Bayesian tree differs from the ML tree and one of the four MP trees in only two ways. First, the sister relationship between *R. t. tucanus* from the south bank of the Amazon river and *R. t. cuvieri* from Peru is strongly supported (98%), to the exclusion of *R. t. cuvieri* from Bolivia. Second, the Bayesian tree places two *R. v. culminatus* as sisters; however, this result is not statistically significant (78%).

Several findings are strongly supported by all three analytical methods (Figs. 3 and 4). The smooth-billed yelping *Ramphastos* always form a strongly supported monophyletic group. *Ramphastos toco* is basal to all other *Ramphastos*. *Ramphastos v. ariel* mtDNA sequences are paraphyletic, with *R. brevis* sister to a monophyletic *R. vitellinus* group (Amazonian *R. v. ariel*, *R. v. vitellinus*, and *R. v. culminatus*) to the exclusion of *R. v. ariel* from southeastern Brazil. This placement of *R. brevis* and *R. v. ariel* from southeastern Brazil is weakly supported by bootstrapping (MP = 60%, ML = 53%) but strongly supported by Bayesian posterior probability (100%). However, the mitochondrial monophyly of *R. v. ariel* (from the Amazon), *R. v. culminatus*, and *R. v. vitellinus* and the mitochondrial paraphyly of *R. v. ariel* are strongly supported by all analytical methods. Amazonian and southeastern Brazilian *R. v. ariel*, though identical in plumage, differ by an average of 3.0% uncorrected sequence divergence. *Ramphastos v. ariel* from southeastern Brazil differs from all other *R. vitellinus* samples by an average of 2.9% uncorrected sequence divergence, whereas the average uncorrected *p*-distance among all other *R. vitellinus* samples is only 0.4%. All three analyses yielded nearly identical results differing only in the relative levels of nodal support. Therefore, I used the pruned tree topology shared by ML, MP, and Bayesian analyses to reconstruct patterns of character evolution in the *Ramphastos* toucans.

Character Reconstruction

The overall CI for characters on the pruned *Ramphastos* tree was 0.48, and the overall RI was 0.57. Individual RIs ranged from 0.00 to 1.00, and individual CIs ranged from 0.33 to 1.00 (Table 5). Consistency and retention indices for more than half the characters were ≤0.50. When mapped onto the *Ramphastos* phylogeny, three of eight characters showed no reversal or homoplasy (Table 5). These three characters (culmen shape, vocalization type, and body size) have relatively high CIs and RIs and exhibited significant phylogenetic signal in the Maddison and Slatkin (1991) randomization test. Culmen shape, vocalization type, and body size characters change state only once on the *Ramphastos* phylogeny (Figs. 5A). For culmen shape and voice, the character transition is from a channel-keel-billed croaking ancestor to a monophyletic group of smooth-billed yelpers. For body size, the transition is from large to small.

Five of eight characters (throat coloration, uppertail-covert coloration, orbital skin coloration, bill pattern, and red breast band) lacked significant phylogenetic signal according to the Maddison and Slatkin (1991) test and had relatively low CIs and RIs (Table 5). For these five characters, I employed both acctran and deltran optimizations to assess the relative frequency of convergence or parallelism (homoplasy) versus shared ancestry (symplesiomorphy) in

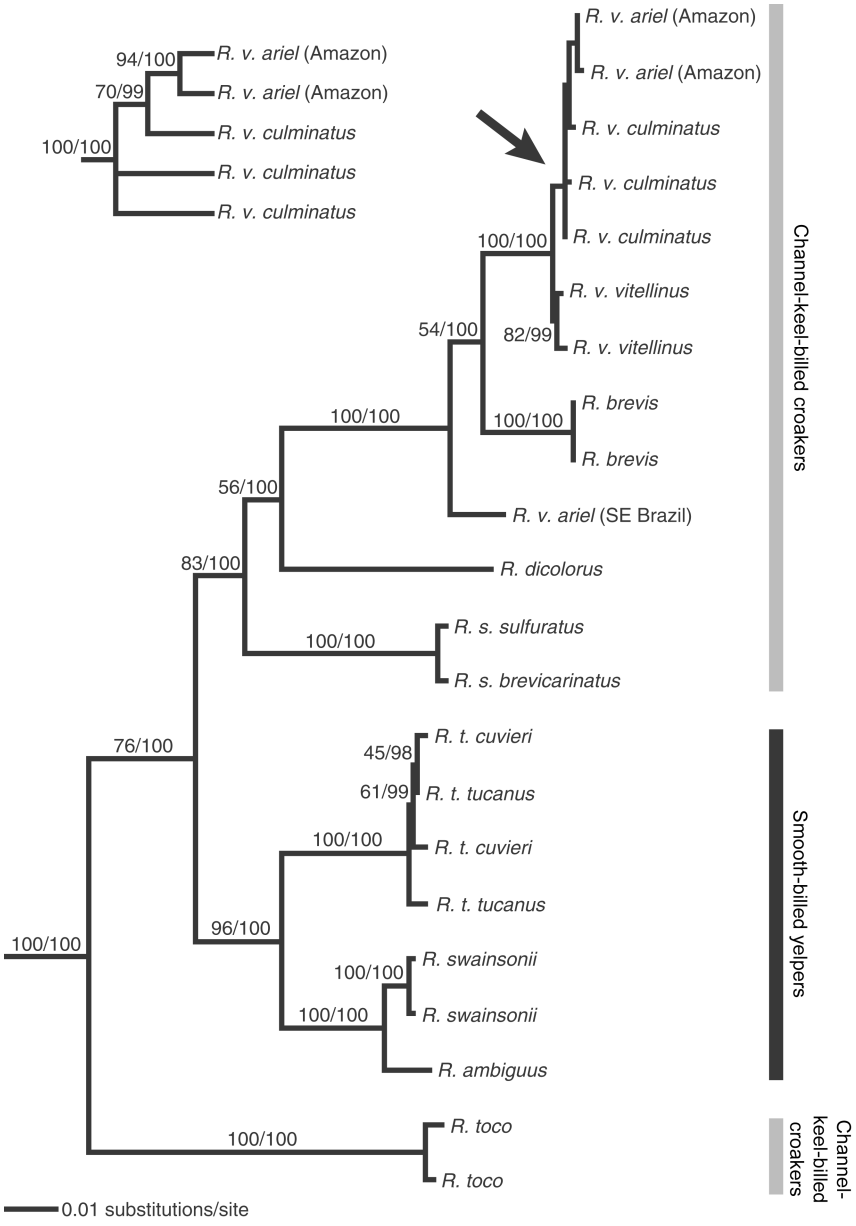

FIG. 4. Phylogram of maximum-likelihood (ML) tree (–lnL = 10975.9482) constructed from the combined mtDNA data set using the TVM + I + G model. The TVM + I + G model includes general time-reversible substitutions (A–C = 1.3219; A–G = 23.8012; A–T = 1.9273; C–G = 0.5643; C–T = 23.8012; G–T = 1.00), unequal base frequencies (A = 0.2942; C = 0.3928; G = 0.1047; T = 0.2083), invariant sites (0.5344), and rate heterogeneity according to a gamma distribution (shape parameter = 1.2173). To the left of each node, numbers indicate levels of ML bootstrap support and Bayesian posterior probabilities. Maximum-likelihood bootstrap values are to the left of the slash, and Bayesian posterior probabilities are to the right of the slash. These values are shown only for nodes where ML bootstrap values are ≥50% or Bayesian posterior probabilities are ≥90%. See inset for support values from the compressed region of the tree marked by the arrow.

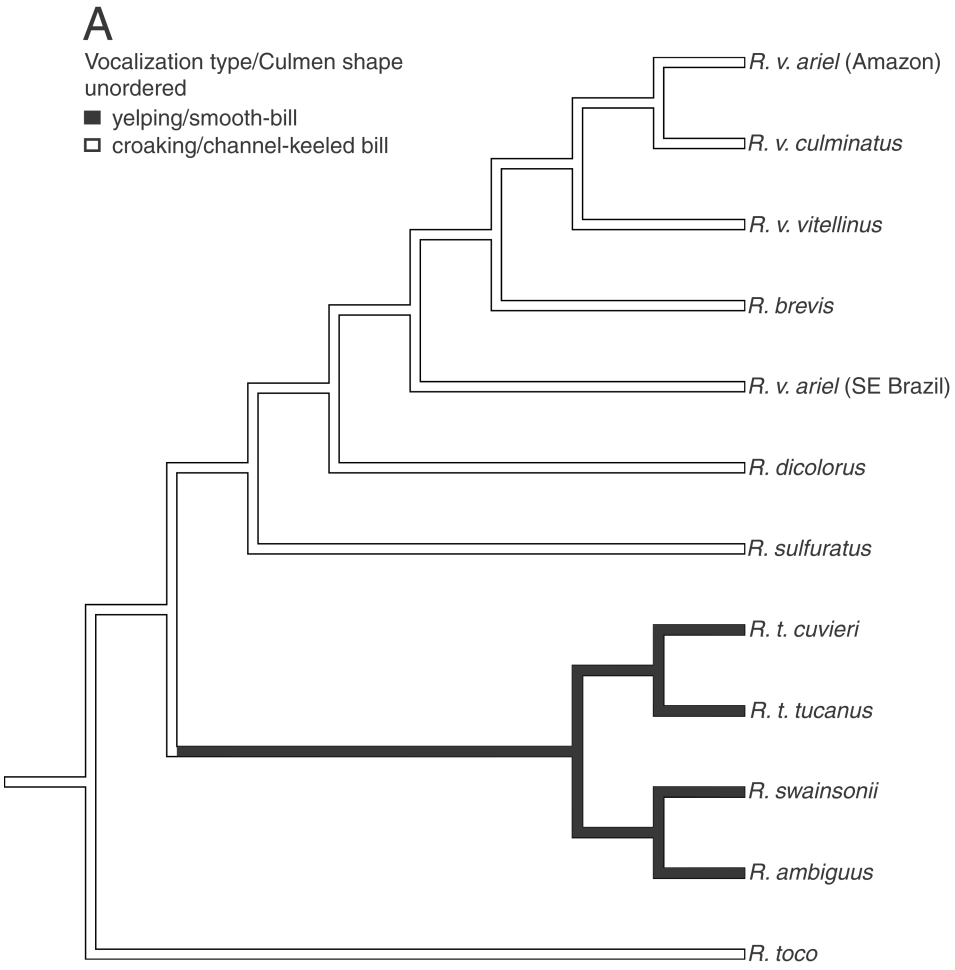

FIG. 5. Acctran reconstruction of (A) vocalization type and culmen shape on pruned *Ramphastos* phylogeny. (Continued on next page.)

TABLE 5. Measures of homoplasy for *Ramphastos* characters. Consistency index (CI) and retention index (RI) are for individual characters mapped onto the pruned *Ramphastos* phylogeny. Signal calculations were done using the method of Maddison and Slatkin (1991).

	Character	CI	RI	Signal (%)[a]
1	Throat coloration	0.33	0.33	38.4
2	Uppertail-covert coloration	0.33	0.50	21.4
3	Orbital skin coloration	0.43	0.00	100.0
4	Bill pattern	0.50	0.50	10.0
5	Red breast band	0.33	0.33	38.0
6	Culmen shape	1.00	1.00	0.3
7	Vocalization type	1.00	1.00	0.5
8	Body size	1.00	1.00	0.2
	Overall index	0.48	0.57	

[a] Values <5% indicate significant phylogenetic signal, and values >5% suggest no significant phylogenetic signal.

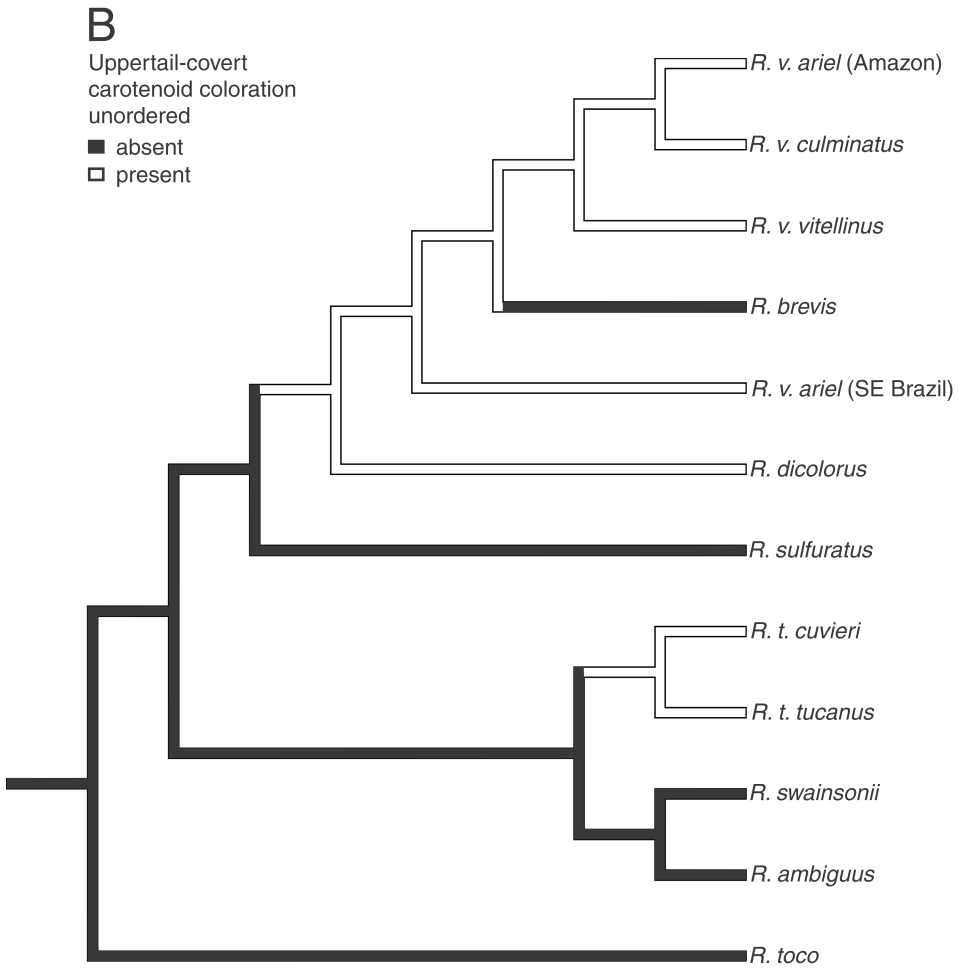

FIG. 5. (Continued.) Acctran reconstruction of (B) uppertail-covert coloration on pruned *Ramphastos* phylogeny.

evolution of the four similar-looking sympatric toucan pairs.

Table 6 summarizes the results of quantifying the amount of similarity in sympatric congeners that is attributable to parallelism or convergence in character state (homoplasy) or retention of a shared ancestral character state (symplesiomorphy). For all characters, character similarity resulting from homoplasy is reconstructed 10 times by the acctran optimization and 7 times by the deltran optimization. Similarity resulting from symplesiomorphy is nearly the same, with eight reconstructed by acctran optimization and five reconstructed using the deltran optimization. The number of symplesiomorphies is roughly equal to the number of homoplasies. Thus, the extreme similarity between sympatric *Ramphastos* pairs is attributable to both homoplasy and symplesiomorphy. This pattern is illustrated in the acctran optimization of uppertail-covert coloration on the *Ramphastos* phylogeny (Fig. 5B). In this example, similarity between species within each of two sympatric pairs of *Ramphastos* is attributable to symplesiomorphic (shared ancestral) uppertail-covert coloration (*R. v. ariel* from southeastern Brazil and *R. dicolorus*, carotenoid uppertail coverts; *R. sulfuratus* and *R. swainsonii*, carotenoids absent from uppertail coverts). In this same reconstruction, similarity in uppertail-covert coloration between species within the other two pairs of sympatric *Ramphastos* is caused by homoplasy

TABLE 6. Numbers of homoplastic (parallel or convergent) and symplesiomorphic (shared ancestral) character reconstructions for five characters of four similar-looking sympatric *Ramphastos* pairs.[a]

	Acctran		Deltran	
Character	Homoplasy	Symplesiomorphy	Homoplasy	Symplesiomorphy
1 Throat coloration	1	3	1[b]	1[b]
2 Uppertail-covert coloration	2	2	2	2
3 Orbital skin coloration	3	1	All equivocal[b]	All equivocal[b]
4 Bill pattern[c]	2	0	2	0
5 Red breast band	2	2	2	2

[a] Similar-looking sympatric pairs include *R. v. ariel* and *R. dicolorus*, southeastern Brazil; *R. sulfuratus* and *R. swainsonii*, Central America; *R. brevis* and *R. swainsonii*, Choco; *R. v. culminatus* and *R. t. cuvieri*, western Amazonia.

[b] Two characters (throat coloration, orbital skin coloration) had equivocal deltran reconstructions that prohibited the assessment of character reconstructions for some or all the sympatric pairs.

[c] Only two pairs of sympatric *Ramphastos* share similar bill patterns (*R. brevis* and *R. swainsonii*, Choco; *R. v. culminatus* and *R. t. cuvieri*, western Amazonia).

(parallelism or convergence) (*R. v. culminatus* and *R. t. cuvieri*, carotenoid uppertail coverts; *R. brevis* and *R. swainsonii*, carotenoids absent from uppertail coverts). For the five coloration characters overall, approximately half the instances of color character similarity are attributable to retention of shared ancestral character states (symplesiomorphy) and half to character convergence or parallelism (homoplasy) (Table 6).

DISCUSSION

PHYLOGENY

Haffer (1974, 1997a, b) postulated relationships for most of the taxa in the genus *Ramphastos*; however, he did not explicitly estimate a phylogeny for the genus using a large number of characters and standard phylogenetic methods. Nevertheless, a comparison of the molecular phylogeny with Haffer's (1974, 1997a, b) hypotheses of relationship is worthwhile. Haffer (1974) stressed differences in voice and bill morphology as indicating a natural division of *Ramphastos* into two groups. He presented branching diagrams (Haffer 1974, 1997a) indicating these two distinct groups, the smooth-billed yelpers and channel-keel-billed croakers (Fig. 6). He hypothesized that taxa within the smooth-billed yelping clade formed two subclades, one including the two subspecies of *R. tucanus* (*tucanus* and *cuvieri*) and the other including *R. swainsonii* and *R. ambiguus*. Within the channel-keel-billed croaking clade, Haffer (1974, 1997a) predicted that *R. dicolorus* was basal to all croakers excluding *R. toco*, which he suggested was basal to all croakers. The other channel-keel-billed croakers were divided into two clades, one with *R. sulfuratus* and *R. brevis* as sisters and the other including all taxa from the *R. vitellinus* group, with *R. v. vitellinus* and *R. v. ariel* sisters and *R. v. culminatus* basal.

My analysis of mtDNA genes produced virtually the same phylogeny as Haffer's (1974, 1997a; Figs. 3, 4, and 6). Most conflicts between topologies occurred at short internodes that were not statistically supported by bootstrapping or Bayesian posterior probabilities. However, one major discrepancy was that phylogenetic estimates using mtDNA sequences strongly support a basal relationship of *R. toco*, the only large-bodied channel-keel-billed croaker, to all other *Ramphastos* toucans. Therefore, character reconstructions (acctran, deltran) on the mtDNA tree suggest that ancestral *Ramphastos* may have been large-bodied channel-keel-billed croakers. Another discrepancy is the placement of *R. sulfuratus* as basal to all channel-keel-billed croakers, excluding *R. toco*; whereas Haffer (1974, 1997a) placed it as sister to *R. brevis*. None of the mtDNA analyses recover *R. brevis* and *R. sulfuratus* as sisters. Rather, mtDNA data place *R. brevis* as basal to all *R. vitellinus* except *R. v. ariel* from southeastern Brazil, but the support for the exact relationships among these taxa differs among analytical methods. Mitochondrial DNA also differs from Haffer (1974, 1997a) in the placement of members of the *R. vitellinus* group. The molecular data place Amazonian *R. v. ariel* as sister to *R. v. culminatus*, with *R. v. vitellinus* basal to both of these taxa. Low divergences and a lack of

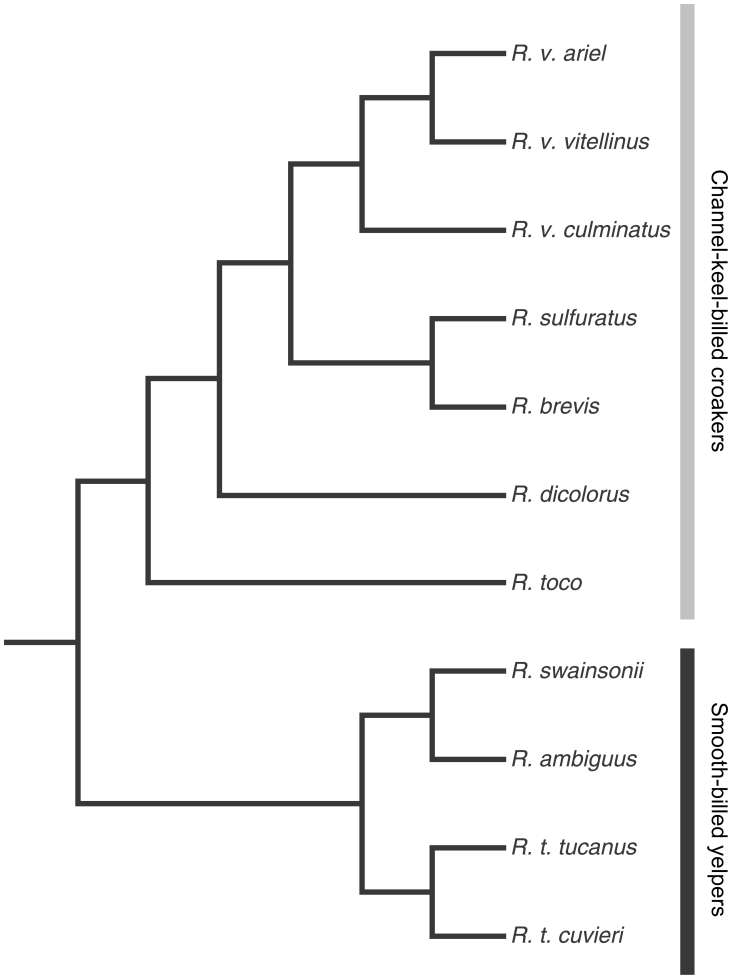

FIG. 6. Phylogeny depicting phylogenetic relationships of species within *Ramphastos* as postulated by Haffer (1974, 1997a).

reciprocal monophyly among Amazonian *R. vitellinus* subspecies may be attributable to ongoing gene flow between these subspecies (see below). All phylogenetic estimation methods using mtDNA data produced a clade of smooth-billed yelpers consisting of two subclades of subspecies within the *R. tucanus* group and the other with *R. swainsonii* and *R. ambiguus* as sisters, as hypothesized by Haffer (1974, 1997a).

Haffer's (1974, 1997a) choice of voice and culmen shape as informative characters for the phylogeny of the *Ramphastos* was fortuitous, because voice and culmen shape have strong phylogenetic signal on the mtDNA phylogeny (Table 5). However, it is not surprising that his reliance on only two characters, voice and culmen shape, did not allow him to predict all relationships correctly, especially those at the tips of the phylogeny. Haffer (1974, 1997a) tacitly assumed that croaking voice and channel-keel-shaped culmen were synapomorphies of members of one *Ramphastos* clade and that yelping voice and a smoothly rounded culmen were synapomorphies shared by another *Ramphastos* clade. However, character reconstructions on the molecular phylogeny (Fig. 5A) indicate that croaking voice and channel-keel-shaped culmen were symplesiomorphic character states (shared ancestral) and that yelping voice and a smoothly rounded culmen were synapomorphies for one *Ramphastos* clade including

R. swainsonii, *R. ambiguus*, *R. t. tucanus*, and *R. t. cuvieri.*

Species Limits

The species limits within the *R. tucanus* group (*R. t. tucanus* and *R. t. cuvieri*) and the *R. vitellinus* group (*R. v. vitellinus*, *R. v. culminatus*, *R. v. ariel*, *R. v. citreolaemus*) are not entirely clear. Some classifications consider subspecies within these two groups separate species (Meyer de Schauensee 1966, 1970; Hilty and Brown 1986; Sibley and Monroe 1990), whereas others do not (e.g. Haffer 1974, Dickinson 2003). In most cases, mtDNA sequence data revealed low divergences among subspecies within each of these two groups of *Ramphastos*. Furthermore, the mtDNA sequences of subspecies within each of these two groups are not reciprocally monophyletic. For example, within the *R. tucanus* group, mtDNA sequences from *R. t. tucanus* and *R. t. cuvieri* do not form reciprocally monophyletic groups and uncorrected mtDNA divergences within the *R. tucanus* group range from 0.2% to 0.6%, which suggests that there is ongoing mtDNA gene flow between *R. t. tucanus* and *R. t. cuvieri*. Carefully documented zones of morphological intergradation around the Amazon River (Haffer 1974, 1997b) are also consistent with this suggestion of ongoing mtDNA gene flow between *R. t. tucanus* and *R. t. cuvieri*.

Mitochondrial DNA sequences from the subspecies of the *R. vitellinus* group also lack reciprocal monophyly, and Amazonian members of the *R. vitellinus* group exhibit relatively low uncorrected mtDNA divergences, ranging from 0.1% to 0.8%, which suggests that there is also ongoing mtDNA gene flow among Amazonian members of the *R. vitellinus* complex. As for the *R. tucanus* group, Haffer (1974, 1997b) carefully documented zones of morphological intergradation between *R. vitellinus* subspecies around the Amazon River, which is consistent with ongoing mtDNA gene flow between *R. v. vitellinus*, *R. v. culminatus*, and Amazonian *R. v. ariel*.

One exception to the low mtDNA divergences found in the *R. vitellinus* group is the divergence between one *R. v. ariel* sampled from southeastern Brazil and all other *R. vitellinus*, which differ by an average uncorrected mtDNA divergence of 2.9%. Phylogenetic analyses strongly support the mitochondrial paraphyly of *R. v. ariel*. The southeast Brazilian mtDNA haplotype is basal to all other *R. vitellinus* mtDNA haplotypes and was not found among 20 *R. vitellinus* sampled from throughout the Amazon basin (J. D. Weckstein and A. Aleixo unpubl. data). Although plumage and soft-part coloration are identical between southeastern Brazilian and Amazonian populations of *R. v. ariel*, these populations are completely allopatric and differ in body size according to measurements reported in Haffer (1974) and Short and Horne (2001). For example, Short and Horne (2001) report that wing chord of male *R. v. ariel* from southeastern Brazil averages 204.2 mm and ranges from 196 mm to 213 mm (n = 10), whereas wing chord of male *R. v. ariel* from Amazonia averages 190.4 mm and ranges from 180 mm to 196 mm (n = 33) (see also figure 16.32 and table 16.11 in Haffer 1974). The relatively high mtDNA divergence between southeastern Brazilian and Amazonian *R. v. ariel*, body size differences between southeastern Brazilian and Amazonian *R. v. ariel*, and lack of the southeastern Brazilian haplotype among many Amazonian samples suggest that populations of *R. v. ariel* in Amazonia may be evolving separately from those in southeastern Brazil. However, more samples are needed from southeastern Brazil to determine whether *R. v. ariel* there carries Amazonian haplotypes.

With the exception of *R. v. ariel* from southeastern Brazil, the relatively low divergences and lack of reciprocal monophyly among mtDNA sequences of subspecies within the *R. tucanus* and *R. vitellinus* groups are consistent with Haffer's (1974) classification, and consistent with the view that subspecies within these two groups are not good species-level taxa. The detection of this lack of monophyly among mtDNA sequences of these subspecies would not have been possible without sampling multiple individuals per species and subspecies, which underscores the need for thorough taxon sampling in phylogenetic studies, as noted by Omland et al. (1999). Future work, involving larger sample sizes, is needed to assess species limits within these groups in more detail.

Character Evolution

Three characters (culmen shape, vocalization, and body size) show significant phylogenetic signal when mapped onto the *Ramphastos*

phylogeny and can, to some extent, indicate phylogenetic relationships of taxa within this genus. In all three cases, there is only a single character transition. Smooth-billed yelping *Ramphastos* are apparently derived.

However, five coloration characters (throat coloration, uppertail-covert coloration, orbital skin coloration, bill pattern, and red breast band) lack significant phylogenetic signal and showed patterns of homoplasy (convergence and parallelism) and symplesiomorphy (retention of ancestral character states). Sympatric *Ramphastos* taxa are not closely related in the phylogeny, and character reconstructions therefore indicate that the extreme similarity in coloration patterns between many sympatric *Ramphastos* pairs is most likely attributable to a combination of convergence or parallelism (homoplasy) and shared ancestral character states (symplesiomorphy). This study and others (Johnson 1999, Crochet et al. 2000, Johnson and Lanyon 2000, Omland and Lanyon 2000) have found relatively high levels of homoplasy in plumage coloration patterns. These high levels of homoplasy caution against the use of plumage and bare-part coloration patterns for estimating avian phylogenies. Similarity in coloration between sympatric *Ramphastos* toucans could be attributable to a number of factors including, but not limited to, ecological competition (Moynihan 1968, Cody 1969, Barnard 1979, Diamond 1982), predator avoidance (Barnard 1979, Diamond 1982, Dumbacher and Fleischer 2001), or adaptation to a common environment (Crochet et al. 2000, Johnson and Lanyon 2000). More work is needed to assess these potential mechanisms for the evolution of similar coloration patterns in pairs of sympatric *Ramphastos* toucans.

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