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Molecular Phylogeny of a Cosmopolitan Group of Woodpeckers (Genus *Picoides*) Based on *COI* and *cyt b* Mitochondrial Gene Sequences

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***Picoides* is the largest genus of woodpeckers and member species are found on most major land masses. Current systematic arrangement of this group, based on morphological, behavioral, and plumage characters, suggests that New World species evolved from a single invasion by a Eurasian common ancestor and that all New World species form a monophyletic group. No clear link has ever been established between the relationships of Old World and New World species other than to infer that the most primitive species is Eurasian. This study employs DNA sequences for two protein-coding mitochondrial genes, cytochrome oxidase I and cytochrome *b*, to reconstruct phylogenetic relationships among all New World species and several Eurasian representatives of the genus *Picoides*. A well-resolved mitochondrial gene tree is in direct conflict with proposed species relationships based on nongenetic characters; monophyly among New World species is rejected, the evolution of New World species likely resulted from as many as three independent Eurasian invasions, and *Picoides* is paraphyletic with two other woodpecker genera, *Veniliornis* and *Dendropicos*. These results strongly suggest that this large, cosmopolitan genus is in need of systematic revision in order to reflect evolutionary history.** © 2002 Elsevier Science

INTRODUCTION

Picoides (*sensu* Short, 1982) is a genus of woodpeckers consisting of nine North American, two South American, 21 Eurasian, and one African species (Short, 1982). The present *Picoides* is a conglomerate of two earlier genera of "pied" woodpeckers, *Picoides* (the "three-toed" woodpeckers) and *Dendrocopos* (the "ladder-backed" woodpeckers); the latter was a large genus consisting of 31 species (Peters, 1948). Thus, the original members of *Picoides* included only two species, *P. tridactylus* and *P. arcticus* (Peters, 1948), both of which have a reduced hallux instead of a distinct fourth zygodactyl toe as in *Dendrocopos* species. Like *Dendrocopos*, these two "three-toed" species have black and white plumage coloration, but males of both species have yellow rather than red display patches on the crown (see Winkler *et al.*, 1995). Delacour (1951) united the two genera into the single genus *Picoides* according to priority in systematic nomenclature claiming that variance in toe length or number is not phylogenetically important among closely allied avian species (see also Short, 1971).

Lester Short made significant and substantial contributions to our knowledge of woodpecker life histories and classification. Short (1982) argued that woodpecker classification should be based on behavior, ecological patterns, and functional morphological characters (e.g., Bock 1963; Short, 1976). These data were used to arrange true woodpeckers into six tribes according to presumed primitive to derived characteristics (Short, 1982). *Picoides* was placed in the tribe Campetherini and is the largest and most cosmopolitan of the woodpecker genera. Although substantial life history data were collected for all 33 members of *Picoides* (e.g., Bock, 1963; Short, 1971, 1973, 1982, and others), Short (1971) applied these data primarily to devise a systematic arrangement for New World *Picoides*. He only superficially addressed the taxonomic relationships among Old World species and demonstrated no clear phylogenetic link between Old World and New World species. Apparently, there are no discussions regarding the phylogenetic arrangement of Old World species of *Picoides* other than to infer that *P. temminckii* and *P. maculatus* have more primitive characteristics (Short, 1982) and the Asian group of *Picoides* that includes *P. minor* has radiated most recently (Short, 1971).

Organisms are united by a single phylogenetic history, and those relationships can only be successfully tracked using characters that are homologous, independently evolving, and heritable. The goal, of course,

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is to reconstruct phylogenetic relationships that accurately reflect evolutionary history and genealogy. Short's substantial efforts toward our current understanding of *Picoides* species relationships is commendable although limited because his phylogenetic hypothesis is based on characters in which independence and homology is uncertain and his phylogenetic methodology was subjective. A phylogeny based on DNA sequences is desirable because it can be generated from a large sample of independent nucleotide sites (Saitou and Nei, 1986; Nei, 1991), homology can be reliably inferred from sequence alignments, and inheritance of DNA is unquestioned.

The mitochondrial genome has served as a phylogenetic workhorse for deciphering evolutionary relationships among avian taxa (e.g., Hedges, 1994; Mindell *et al.*, 1997; Harlid *et al.*, 1998; Nunn and Stanley, 1998; Johnson and Lanyon, 1999; Mindell *et al.*, 1999; Sheldon *et al.*, 2000; and others). High resolution among recently diverged, closely related species is due to the following desirable properties of mitochondrial DNA for resolving the gene tree in phylogenetic analysis: (1) adequately rapid nucleotide substitution rates that mark points of common ancestry without obliterating accrued synapomorphies (Lanyon, 1988); (2) similar substitution rates across lineages; and (3) unchanging bias in nucleotide composition across lineages (Irwin *et al.*, 1991).

The purpose of this study is to test Short's phylogenetic hypothesis of the species relationships of *Picoides* woodpeckers and establish the evolutionary connection between Old World and New World species using rigorous phylogenetic analysis based on two mitochondrial protein-coding genes, cytochrome oxidase I (*COI*) and cytochrome *b* (*cyt b*). The results of this work will likely necessitate systematic revision of the genus *Picoides* allowing more thorough and accurate study of the evolutionary history of this large, cosmopolitan group of woodpeckers.

MATERIALS AND METHODS

Total DNA was isolated from fresh frozen muscle, liver, or kidney tissues or tissues preserved in ethanol or EDTA using proteinase K/SDS methods adapted from Maniatus *et al.* (1982) or Qiagen DNeasy Tissue Kit according to manufacturers specifications. Specimens used in this study are listed in Table 1.

Two mitochondrial protein-coding genes, *COI* and *cyt b*, were amplified by the polymerase chain reaction (PCR) following the methods of Kocher *et al.* (1989), Edwards *et al.* (1991), Moore and DeFilippis (1997), and DeFilippis and Moore (2000). Primers used for amplification and subsequent DNA sequencing are listed in Table 2. Amplification products were cleaned using Promega Wizard PCR Prep Kit.

Double-stranded PCR products for both *COI* (1551 of 1551 bases) and *cyt b* (1029 of 1143 bases) were sequenced using either an Amersham-Pharmacia ALF automated sequencer with a Thermosequenase Reaction Kit or an Applied Biosystems ABI 100 model 377 automated sequencer with the Big Dye Terminator Reaction through the Wayne State University Molecular Core Facility. A single strand (the light strand) was sequenced for each of two specimens, if available, of each species. Sequences were aligned by eye using ESEE (Cabot and Beckenbach, 1989). Primers permitted substantial overlap of sequenced fragments allowing clarification of ambiguous nucleotides by direct comparison of aligned sequences. Sequencing two specimens tests for contamination of PCR products as conspecific sequences should have very low intraspecific nucleotide divergence and pair as sister taxa in phylogenetic analysis. Statistical analysis of sequence data was performed using MEGA version 1.01 (Kumar *et al.* 1993), and phylogenetic analysis was performed in PAUP* beta version 4.0 (Swofford, 1998).

A maximum-likelihood (ML) analysis was performed according to a sequential optimization approach modified from Frati *et al.* (1997; see also Steppan *et al.*, 1999) to increase computational efficiency with large data sets. The procedure is summarized in Table 3. Trees generated from a neighbor-joining (NJ) analysis (Saitou and Nei, 1987) and a maximum parsimony (MP) analysis each served as a user-defined working topology on which four different substitution models were evaluated under a ML criterion: Jukes-Cantor (JC) (Jukes and Cantor, 1969), Kimura Two-Parameter (K2P) (Kimura, 1980), Hasegawa-Kishino-Yano (HKY85) (Hasegawa *et al.*, 1985), and general time-reversible (GTR) (e.g., Yang, 1994). Six GTR rate matrix parameters, the proportion of invariable sites (I), and the gamma distribution shape parameter (α) for rate variation of nucleotide sites were simultaneously optimized under each substitution model and each working topology. A log likelihood test (see Goldman, 1993) determined the best of the four nested substitution models for explaining the data under each working topology. Degrees of freedom were calculated as the difference in the number of estimated parameters between two models.

The statistically superior substitution model under each working topology was then evaluated in combination with the parameters I and α. Each working topology was evaluated under a ML criterion for four different substitution/rate variation models: the best substitution model assuming equal rates, the best model+I, the best model+α, and the best model+I+α. A log likelihood test determined the best of these four substitution/rate variation models for explaining the data under each working topology.

For each working topology, the best substitution/rate variation model and its respective parameter estimates served as the optimized model to search for the

TABLE 1

List of Species (Order Piciformes, Family Picidae)

Species	Common name[b]	Locale	Museum[c]	Voucher number	Intraspecific divergence[d]		Template sequence[e]	
					COI	*cyt b*	*COI*	*cyt b*
Subfamily Picinae								
Tribe Campetherini								
Picoides albolarvatus	White-headed WP	California, USA	WSU	86W-14.1	0	0.31		
P. albolarvatus	White-headed WP	California, USA	WSU	86W-14.5	—	—	X	X
P. arcticus	Black-backed WP	Montana, USA	WSU	86W-16.2	0.34	0.09		X
P. arcticus	Black-backed WP	Montana, USA	WSU	86W-16.3	—	—	X	
P. borealis	Red-cockaded WP	Florida, USA	FSU	209-1	1.01	0.60	X	X
P. borealis	Red-cockaded WP	Florida, USA	FSU	314-3.1	—	—	NA	
P. borealis	Red-cockaded WP	Florida, USA	FSU	314-3.2	—	—		
P. canicapillus	Grey-capped WP	Primorskiy Kray, Russia	UW	51079			X	X
P. kizuki	Pygmy WP	Sakhalinskaya Oblast, Russia	UW	47374	0.07	0.10	X	
P. kizuki	Pygmy WP	Sakhalinskaya Oblast, Russia	UW	47379	—	—		X
P. leucotos	White-backed WP	Moscovskaya Oblast, Russia	UW	49580	0	0.20		
P. leucotos	White-backed WP	Moscovskaya Oblast, Russia	UW	49608	—	—	X	X
P. lignarius	Striped WP	Santa Cruz, Bolivia	LSU	6593			X	X
P. maculatus	Philippine WP	Philippines	USNM	607368			X	X
P. major	Great Spotted WP	Irkutskaya Oblast, Russia	UW	51700	0.14	0.59	X	X
P. major	Great Spotted WP	Krasnoyarskiy Kray, Russia	UW	51755	—	—		
P. minor	Lesser Spotted WP	Khabarovskiy Kray, Russia	UW	47225	0.33	0	X	X
P. minor	Lesser Spotted WP	Khabarovskiy Kray, Russia	UW	47226	—	—		
P. mixtus	Checkered WP	Provincia de Corrientes, Argentina	UW	810	0.21	0.10	X	
P. mixtus	Checkered WP	Provincia de Corrientes, Argentina	UW	816	—	—		X
P. nuttallii	Nuttall's WP	California, USA	WSU	86W-13.1	0.37	0	X	
P. nuttallii	Nuttall's WP	California, USA	WSU	86W-13.3	—	—		X
P. pubescens	Downy WP	Alabama, USA	WSU	86W-2.3	0.13	0.50		X
P. pubescens	Downy WP	Texas, USA	WSU	86W-5.5	—	—	X	
P. scalaris	Ladder-backed WP	New Mexico, USA	WSU	86W-8.2	0	0.20		X
P. scalaris	Ladder-backed WP	Arizona, USA	WSU	86W-11.7	—	—	X	
P. stricklandi	Strickland's WP	Arizona, USA	WSU	88W-2.2		0.23	NA	
P. stricklandi	Strickland's WP	Arizona, USA	UA	16860		—	X	X
P. tridactylus	Three-toed WP	Sakhalinskaya Oblast, Russia	UW	47015	0.14	0.20		
P. tridactylus	Three-toed WP	Vologdaskaya Oblast, Russia	UW	49797	—	—	X	X
P. villosus	Hairy WP	Arizona, USA	WSU	86W-10.7	0.32	2.39		
P. villosus	Hairy WP	California, USA	WSU	86W-14.4	—	—	X	X
Dendropicos fuscescens	Cardinal WP	Kwa Zulu Natal Province, S. Africa	UW	471			X	X
D. griseocephalus	Olive WP	Iringa Ndundulu Mts., Tanzania	UC	p815			X	X
Subfamily Picinae								
Tribe Colaptini								
Veniliornis callonotus	Scarlet-backed WP	Lambayeque, Peru	LSU	5178				
V. nigriceps	Bar-bellied WP	La Paz, Bolivia	LSU	8176				
Colaptes auratus[a]	Northern Flicker	Kentucky, USA	WSU	86-1.8				
Piculus rubiginosus[a]	Golden-olive WP	Lambayeque, Peru	LSU	5222				
Subfamily Picinae								
Tribe Melanerpini								
Melanerpes carolinus[a]	Red-bellied WP	Kentucky, USA	WSU	86W-1.4				
Sphyrapicus varius[a]	Yellow-bellied SS	Michigan, USA	WSU	86W-14.8				
Subfamily Picinae								
Tribe Campephilini								
Dryocopus pileatus[a]	Pileated WP	Texas, USA	WSU	86W-3.4				
Subfamily Picumninae								
Tribe Picumnini								
Picumnus aurifrons[a]	Bar-breasted Piculet	Santa Cruz, Bolivia	LSU	18254				

[a] Outgroup species; *COI* and *cyt b* sequences were obtained from DeFilippis and Moore (2000) and Moore and DeFilippis (1997), respectively.

[b] WP, Woodpecker, SS, sapsucker.

[c] FSU, Florida Stata University (F. James); LSU, Louisiana State University Museum of Natural Science; UA, University of Arizona; UC, University of Copenhagen Avian Blood Bank; USNM, United States National Museum; UW, Burke Museum at University of Washington; WSU, Wayne State University (W. S. Moore).

[d] Percent sequence divergence between at least two conspecific specimens.

[e] A single specimen for a species serves as the template sequence for combining conspecific specimens. NA, not available; the specimen could not be sequenced.

TABLE 2

***COI* and *cyt b* Primers for Amplification and Sequencing**

COI primers	Sequence	Source[b]
L6615	5′-CCTCTGTAAAAAGGACTACAGC-3′	D. P. Mindell
L6772	5′-TTAGCCTCCTCATTCGAGCAGAATTGGG-3′	V. R. DeFilippis
L6958	5′-AATAACATAAGCTTCTGACT-3′	D. P. Mindell
L7165	5′-ACCGCCATCAACATAAAACCCCC-3′	D. P. Mindell
L7444	5′-TACTCCGGAAAAAAAGAACC-3′	D. P. Mindell
L7551	5′-CCGTAGGAATGGACGTTGACACCCGAGC-3′	C. E. Schous
H7539	5′-CATCTGTGGGCTCGGATGAAATGTAG-3′	V. R. DeFilippis
L7945	5′-CCCCAACACTTCCTCTGCCTAGC-3′	A. C. Weibel
H8191	5′-CCAICITIIGAGGGTTCGATTCCTTCC-3′	V. R. DeFilippis
cyt b primers		
B1 (L14841=CBL14990)	5′-GCTTCCATCCAACATCTCAGCATGATG-3′	Kocher *et al.* (1989)
B2 (H15149=CBH15301)	5′-GCAGCCCCTCAGAATGATATTTGTCCTCA-3′	Kocher *et al.* (1989)
C (CBL15311)	5′-GCAAGCTTCTACCATGAGGACAAATATC-3′	S. Pääbo
D (L15424=CBL15573)	5′-ATCCCATTCCACCCATACTACTC-3′	Edwards *et al.* (1991)
Ed9 (L15609=CBL15609)[a]	5′-ATCCTACGCTCCATCCCCAACAAACT-3′	Edwards *et al.* (1991)
E (H15547=CBH15695)	5′-AATAGGAAGTATCATTCGGGTTTGATG-3′	Edwards *et al.* (1991)
Ed10 (H15767=CBH15767)	5′-ATGAAGGGATGTTCTACTGGTTG-3′	Edwards *et al.* (1991)
CBH16065	5′-GGAGTCTTCAGTCTCTGGTTTACAAGAC-3′	W. S. Moore

Note. COI primers are named according to the mitochondrial strand of synthesis (L = light and H = heavy) followed by the 3′ nucleotide binding site number in the chicken mitochondrial genome (Desjardins and Morais, 1990). *cyt b* primer names in parentheses beginning with L/H follow the naming scheme of Edwards *et al.* (1991) and those beginning with CBL/CBH follow the same naming scheme as *COI* primers.

[a] Modified primer sequence from Edwards *et al.* (1991).

ML tree using a heuristic search, tree bisection and reconnection (TBR) branch swapping, and 10 random addition replicate data sets. All trees (NJ, MP, and ML) were rooted with *Picumnus aurifrons* (a piculet), and bootstrap analyses were performed on NJ and MP trees with 1000 replicate data sets.

RESULTS

Five of the 19 species in the tribe Campetherini were sequenced in overlapping fragments for one specimen because of limited tissue availability, and a second specimen of two species could not be sequenced for the *COI* gene (Table 1). Although *COI* and *cyt b* sequences for *Veniliornis callonotus, V. nigriceps,* and two specimens of *Picoides villosus* were generated from earlier works (*COI,* DeFilippis and Moore, 2000; *cyt b,* Moore and DeFilippis, 1997), these specimens were resequenced to improve the completeness and extend the length of the sequence; minor differences in sequences of these specimens were detected between the previous studies and this study. Sequences for the remaining 12 species were obtained from two specimens (three specimens for *P. borealis*), and all conspecific specimens

TABLE 3

Sequential Optimization for Maximum-Likelihood Phylogenetic Analysis

Step 1: Generate user-defined working topology.	Step 2: Evaluate each working topology for each substitution model using ML criterion.[a]		Step 3: Evaluate each working topology for each substitution/rate variation model using ML criterion.[b]		Step 4: ML topology search.[c]
NJ	JC	Identify the best substitution model based on log L ratio test	Best substitution model + equal rates	Identify the best rate variation model based on log L ratio test	NJ-based ML parameter set
MP	K2P		Best substitution model + I		
	HKY85		Best substitution model + α		MP-based ML parameter set
	GTR		Best substitution model + I + α		

[a] GTR rate matrix parameters, I, and α are simultaneously estimated under each substitution model and each user-defined working topology. JC, Jukes and Cantor (1969), model; K2P, Kimura (1980) Two-Parameter model; HKY85, Hasegawa *et al.* (1985) model; and GTR, General Time-Reversible model (e.g., Yang, 1994).

[b] A substitution/rate variation model = a substitution model + estimated rate parameters from step 2.

[c] A ML tree is generated from parameters estimated for the best rate variation model based on each working topology (NJ and MP); note that MP analysis may produce more than one working topology.

TABLE 4

Analysis of *COI* and *cyt b* Sequence Data for Ingroup Woodpeckers

		COI	*cyt b*
Total nucleotide sites		551 bases	1029 bases
Variable sites		532 sites	378 sites
Transition:transversion[a]		6.9 (ML estimate = 5.7)	14.2 (ML estimate = 8.3)
Variable sites of total sites		34.3%	36.7%
Mean % nucleotide composition ± 1 standard deviation	A	26.1 ± 0.52	24.6 ± 0.59
	C	32.5 ± 0.83	35.9 ± 0.51
	G	16.6 ± 0.46	13.3 ± 0.35
	T	24.8 ± 0.87	26.2 ± 0.74

	% total sites	% variable sites	% total sites	% variable sites
Synonymous substitution sites	29.7	86.7	30.7	83.6
Nonsynonymous substitution sites	4.6	13.4	6.0	16.4
Synonymous twofold degenerate sites	11.9	34.6	12.4	33.9
Nonsynonymous twofold degenerate sites	0.39	1.1	0	0
Synonymous fourfold degenerate sites	14.9	43.4	14.0	38.1
Nonsynonymous fourfold degenerate sites	0	0	0	0
Other variable sites	7.2	20.9	10.3	28.0

[a] Ratio was estimated from independent pairs of recently diverged taxa, and values in parentheses were estimated by maximum-likelihood (see text).

paired as sister taxa in preliminary phylogenetic analysis, indicating that these sequences are not contaminates. *COI* and *cyt b* sequences produced from this study are available from GenBank (accession numbers AF394273–AF394306 and AF389302–AF389337, for *COI* and *cyt b*, respectively).

With one exception, intraspecific sequence divergence for the 12 species pairs was at most 1.01% for both the *COI* and *cyt b* genes (Table 1). The exception occurred in *Picoides villosus* having 2.39% intraspecific sequence divergence for the *cyt b* gene. Because of low intraspecific divergence and the large number of taxa used in this study, sequences for pairs of specimens for a given species were combined to form a single "synthetic" sequence according to the following method. First, the best sequence of the two specimens was selected to serve as the "template" sequence based on completeness, fewest ambiguous sites, and greatest overlap of independently sequenced fragments (Table 1). Next, missing data in the template sequence were filled using the homologous overlapping sequence from the second specimen of the same species. Homologous sites with different nucleotides across conspecific sequences were considered ambiguous and were scored as missing data in the synthetic sequence. The resultant alignment of synthetic sequences is available from Weibel (2001).

COI and *cyt b* have nearly the same proportion of variable sites, but *cyt b* has a higher transition to transversion ratio (Table 4). Overall, the percent nucleotide base composition is very similar for the two genes (Table 4). Substitution rates between *COI* and *cyt b* for five independent pairs of recently diverged ingroup taxa (see Fig. 1) were compared using a *Z* test statistical analysis devised by DeFilippis and Moore (2000). However, an error was detected in the published test statistic formula; the correct formula for *Z* is the quotient of the sum of proportions of nucleotides that differ among pairs of taxa for *COI* and *cyt b* genes and the *square root* of the sum of estimated binomial variances for each gene. No statistically significant differences were detected at the 0.05 level of significance for overall proportion of variable sites ($Z = 0.6000$), synonymous substitution rates ($Z = 0.4583$), or nonsynonymous substitution rates ($Z = 0.7333$). Because *COI* and *cyt b* sequences are evolving identically among *Picoides* species, the two data sets were combined to form an aggregate DNA sequence data set of 2580 bases on which phylogenetic analysis was performed (see Bull *et al.*, 1993; Huelsenbeck *et al.*, 1996).

MP analysis was performed using equally weighted characters, a heuristic search, TBR branch swapping, and 30 random addition replicate data sets. A preliminary NJ topology was generated using GTR distances (see Waddell and Steel, 1997). The rate parameters I and α were simultaneously estimated from this preliminary NJ topology under a ML criterion. A second NJ analysis was performed using GTR distances and the estimated rate parameters (I and α) to improve branch length estimates; this second NJ tree served as the distance-based working topology.

Figure 1a shows the NJ tree, and Fig. 1b shows the single MP tree. Both topologies show good resolution with bootstrap values of at least 70% at most nodes. Hillis and Bull (1993) used a four-taxon simulation model to assess that bootstrap values of at least 70%

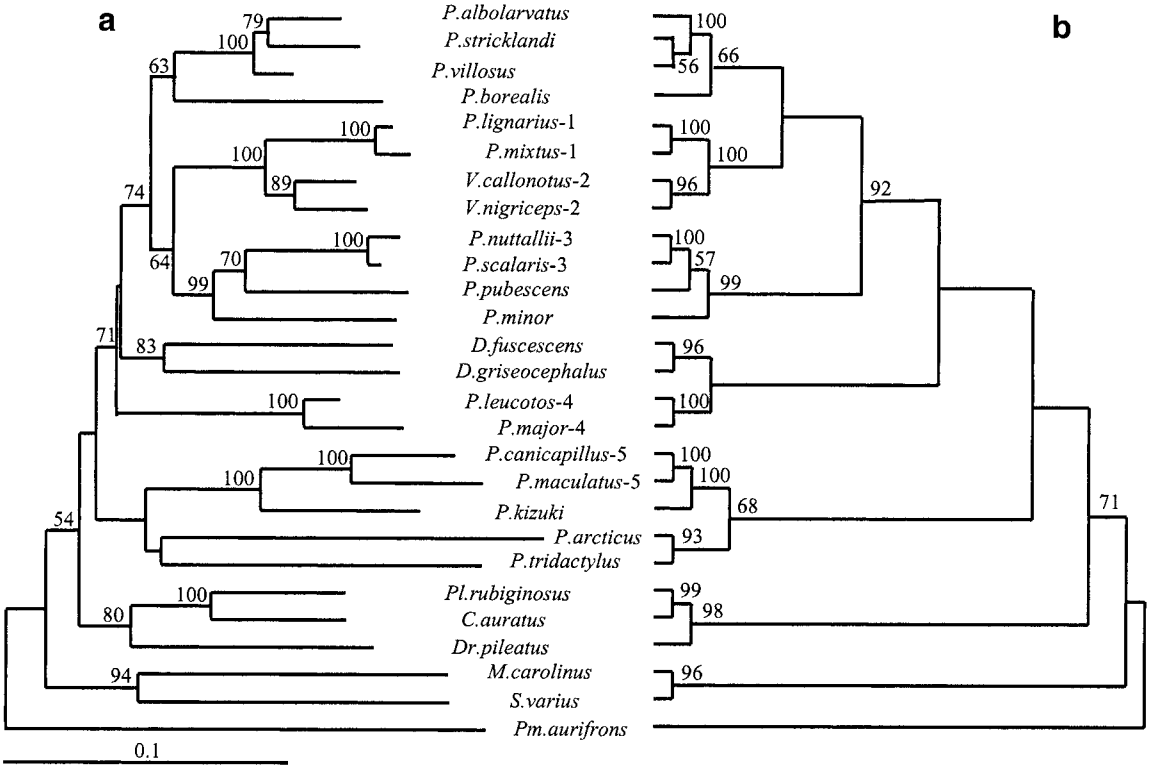

FIG. 1. Phylogenetic reconstruction of *Picoides* showing paraphyly with the groups *Veniliornis* and *Dendropicos* using (a) the neighbor-joining algorithm with GTR distances (minimum evolution score = 2.00075) and (b) the maximum parsimony criterion assuming equal weighting of characters (length = 3619, CI = 0.393, RI = 0.416, RC = 0.164). Bootstrap values of at least 50% support for 1000 replicate data sets are shown at nodes. Outgroup includes representative species of woodpecker genera *Piculus, Colaptes, Dryocopus, Melanerpes,* and *Sphyrapicus*. Both trees are rooted with the piculet *Pm. aurifrons*. Ingroup species pairs labeled 1–5 are independent, recently diverged pairs of taxa used for estimating transition to transversion ratios according to Moore and DeFilippis (1997).

reflect a probability of 95% that the node is real; this result is adopted here to infer statistical support of nodes in phylogenetic trees. Only deeper nodes and the shortest internodes are not statistically supported by bootstrap values. *Picoides* is paraphyletic with the South American *Veniliornis* and the African *Dendropicos* genera. *Veniliornis* is clearly sister to the two South American *Picoides* species (*P. lignarius* and *P. mixtus*). The relationship of *Dendropicos* is unclear but appears to be related to two Eurasian *Picoides* species (*P. leucotos* and *P. major*). One Eurasian species (*P. minor*) is sister to a group of North American species of *Picoides,* and the North American "three-toed" woodpeckers (*P. arcticus* and *P. tridactylus*) are more closely related to Eurasian species than to other North American species. Rooting with a nonwoodpecker species (a piculet), a member of the subfamily Picumninae which is sister to the woodpecker subfamily Picinae (Short, 1982), results in the exclusion of all outgroup species from the ingroup except for the two paraphyletic groups *Veniliornis* and *Dendropicos*. The NJ and MP topologies are similar with minor differences. The NJ tree (Fig. 1a) shows the South American species (*P. lignarius, P. mixtus,* and *Veniliornis* spp.) grouping with "small" *Picoides* species (*P. nuttallii, P. scalaris, P. pubescens,* and *P. minor*), whereas these South American forms group with "large" *Picoides* species (*P. albolarvatus, P. stricklandi, P. villosus,* and *P. borealis*) in the MP tree (Fig. 1b). However, neither topology is strongly supported by bootstrap proportions at these nodes.

The GTR + I + α model was superior for explaining the data under both the NJ and MP working topologies. Parameter estimates (GTR rates, I, and α) from each working topology were used to search for the ML tree under this model. Thus, two separate ML analyses were performed. Searches for the ML tree found the same topology for both sets of parameters, and the NJ-based ML tree, having a slightly higher likelihood than the MP-based ML tree ($-\ln L = 18499.01520$ vs $-\ln L = 18499.05028$), is shown in Fig. 2a. The ML tree shows paraphyly in *Picoides* with *Veniliornis* and *Dendropicos,* and like the NJ tree, the ML tree groups South American species with the "small" *Picoides* species. One unexpected novelty of the ML tree is that the *Piculus–Colaptes–Dryocopus* group does not remain part of the outgroup and effectively splits a Eurasian group of *Picoides* that includes the two North American "three-toed" woodpecker species from other *Pi-*

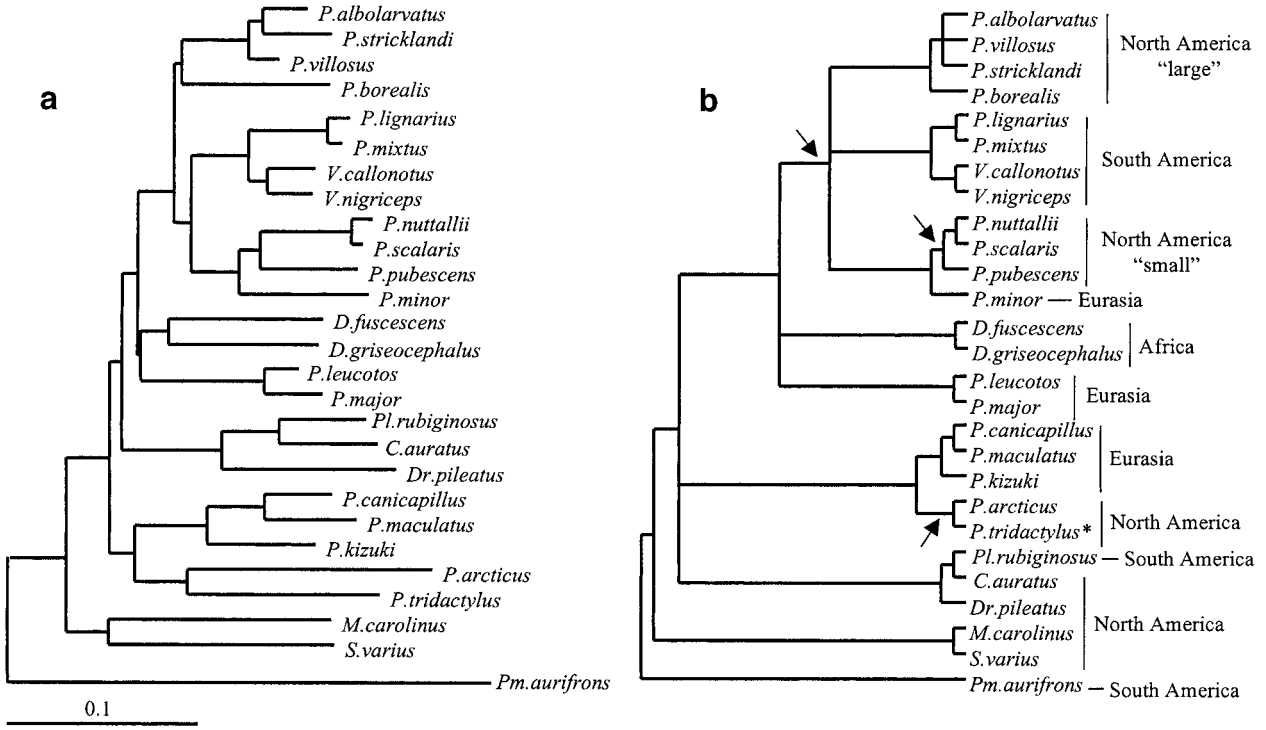

FIG. 2. (a) The resulting topology of a maximum-likelihood search using parameters for the GTR + I + α model estimated from the neighbor-joining tree topology. (b) The strict consensus of the neighbor-joining, maximum parsimony, and maximum-likelihood topologies. General global distributions of species are indicated. (*) *Picoides tridactylus* is also found in Eurasia. Arrows show points of Eurasian invasion into the New World; a New World to Eurasian invasion may have occurred in the *P. minor* group.

coides. This relationship does not occur in either the NJ or MP topologies, however, neither topology shows statistical support for the exclusion of the *Piculus–Colaptes–Dryocopus* group from the ingroup.

It is clear that *COI* and *cyt b* have evolutionary properties that are sufficient to resolve most but not all recent bifurcation events in *Picoides*. Further, the three phylogenetic methods generated slightly different species arrangements, though a Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) did not show a statistically significant difference between the ML topology and the NJ ($P = 0.45$) or the MP ($P = 0.33$) topologies (see Goldman *et al.*, 2000). Thus, to arrive at a best estimate of evolutionary relationships among the species under study based on *COI* and *cyt b* sequence data, a strict consensus tree was generated that contains only those groups appearing in all three rival topologies. In general, nodes that are not statistically supported by bootstrap values (i.e., deep evolutionary splits) are collapsed to reflect ambiguity among the three trees; the collapsed tree is shown in Fig. 2b. Poor resolution persists among the two groups of North American species of *Picoides* (including the Eurasian species *P. minor*) and South American forms, yet the three groups are distinctly grouped together. The relationship between two Eurasian species of *Picoides* and the two African *Dendropicos* species is not clear, however, the paraphyly of *Picoides* with *Dendropicos* remains evident. The relationship of the *Piculus–Colaptes–Dryocopus* group with the ingroup inferred in the MP tree remains ambiguous in the collapsed tree.

DISCUSSION

Picoides is the largest genus of woodpeckers, and accurate estimation of phylogenetic relationships of such a large group requires substantial taxon sampling (Poe, 1998; Rannala *et al.*, 1998). In this study, all North American and South American species are represented, but only six of 22 of the remaining species were available from museum tissue collections. However, the overall resolution of species relationships in the *COI* + *cyt b* gene trees as shown by high bootstrap values (Felsenstein, 1985a; Felsenstein and Kishino, 1993), indicating confidence at nodes (Hillis and Bull, 1993), and the relatively short terminal branches in all topologies (Rannala *et al.*, 1998), particularly among New World species, suggest that the number of species of *Picoides* united by a most recent common ancestor is adequate for accurate phylogenetic resolution.

Results of phylogenetic analysis can be affected by choice of outgroups as the root position of the ingroup may be misidentified (Smith, 1994; Graybeal 1998). Here, outgroup taxa were selected that were proposed

as close relatives to *Picoides* or that would break up long branches leading to the ingroup. The work by Moore and DeFilippis (1997), DeFilippis and Moore (2000), and Prychitko and Moore (2000) provided good indications of appropriate outgroup species and revealed a close sister species relationship between *P. villosus* and two species of *Veniliornis,* even though Short (1982) put the two genera in different tribes; *Veniliornis* is in the tribe Colaptini and *Picoides* is in the tribe Campetherini (see Table 1). *Picoides obsoletus,* the only African species in the genus, was suspected by Goodwin (1968) to be in the African genus *Dendropicos*. Thus *Dendropicos* was included in this study because of its proposed sister genus relationship with *Picoides*. All methods of phylogenetic analysis performed in this study reveal that *Veniliornis* and *Dendropicos* are clearly part of the *Picoides* ingroup and therefore are not considered here as outgroup taxa.

Although this study indicated no statistically significant difference in substitution rates, the *cyt b* gene tends to evolve at a slightly faster rate than the *COI* gene (e.g., Brown, 1983; Moore and DeFilippis, 1997). This is particularly evident in transition to transversion ratios estimated by both the independent pairs sampling method (Moore and DeFilippis, 1997) (see numerically paired species in Fig. 1) and ML estimation and in synonymous substitution rates. A larger GC bias is also apparent in the *cyt b* gene. These trends in the data may explain why the efficacy of *cyt b* to resolve older splits diminishes when the gene is analyzed alone and analysis of *COI* alone is more effective at resolving these older divergence events (not shown). Most of the phylogenetic signal is contained in synonymous substitutions and pooling the two data sets results in a larger sample size that improves statistical inference at particular nodes of a tree (Zardoya and Meyer, 1996; DeFilippis and Moore, 2000). Thus, combining *cyt b* and *COI* data improves the overall resolution of species relationships in both recent and older splits.

Disparities among the NJ, MP, and ML topologies occur at short internodes where the amount of nucleotide data may be insufficient for resolution and at nodes that fall deep in the tree for which rapid substitution rates in either *cyt b* or *COI* erase informative characters that might establish well-supported relationships (Saitou and Nei, 1986; Moore and DeFilippis, 1997; DeFilippis and Moore, 2000). In virtually all cases, disparities among topologies involve nodes without significant bootstrap support. For example, the relationships among the three New World species groups differ between the MP topology and the NJ and ML topologies. Previous works (DeFilippis and Moore, 2000; and Prychitko and Moore, 2000) showed strong support for a *Veniliornis–P. villosus* (a "large" species) sister relationship. However, these studies included a single representative of the genus *Picoides* and excluded important basal species needed to resolve clearly the relationships between *Veniliornis* and New World *Picoides*. Also, the bizarre placement of the *Piculus–Colaptes–Dryocopus* outgroup within the *Picoides* ingroup occurs only in the ML analysis. This relationship does not occur in either the NJ or MP analyses, but statistical support for the expected outgroup relationship is not found in either topology. Recognizing the limitations of *COI* and *cyt b* sequence data to resolve fully deep splits (see Moore and DeFilippis, 1997; DeFilippis and Moore, 2000), the most conservative estimation of phylogenetic relationships in the genus *Picoides,* in the context of these data, is to collapse unsupported branches.

Short (1971) proposed that New World *Picoides* evolved from a single Eurasian common ancestor that gave rise to two distinct groups. One group consists of the "three-toed" woodpecker lineage and the lineage of "large" New World *Picoides* (*P. albolarvatus* (*P. villosus, P. stricklandi*)). The second group consists of the lineage of the two South American species and the lineage of "small" New World *Picoides* (*P. borealis* (*P. pubescens* (*P. nuttallii, P. scalaris*))). Interestingly, *P. borealis* is a relatively large bird that, like other "large" *Picoides* woodpeckers, prefers pine and pine–oak habitat.

The collapsed mitochondrial gene tree (Fig. 2b) clearly refutes Short's hypothesis on a number of specifics. First, "large," "small," and South American groups of *Picoides* are united by a single common ancestor with *P. borealis* grouping with the "large" rather than the "small" species as predicted by Short (1971). *Picoides minor,* a Eurasian species, is grouped with the "small" North American species. Although many Eurasian species have not been included in this study, it is clear that the common ancestor of the "small" species and *P. minor* gave rise to a recent North American rather than Eurasian radiation. Second, member species of New World *Picoides* are not monophyletic as proposed by Goodwin (1968) and Short (1971) because all analyzed Eurasian species are interspersed among New World species. In fact, New World *Picoides* may have evolved from at least three Eurasian invasions (see Fig. 2b); a retreat from the New World to Eurasia by the ancestor to *P. minor* is also plausible. It is clear that the "three-toed" woodpeckers descended from the most basal lineage of all *Picoides* species studied. Their close relationship with *P. maculatus,* a species proposed by Short (1982) to be the most primitive in the genus, further refutes Short's hypothesis of a single Eurasian invasion into North America and the monophyly of New World species. This research provides the first link between Old World and New World *Picoides* (exclusive of the holarctic distribution of *P. tridactylus*), and it appears that the red-vented *Picoides* (*P. major* and *P. leucotos*) (see Winkler *et al.*, 1995) are the closest Eurasian relatives to the group of North Amer-

ican and South American *Picoides*. A perspective of the historical biogeography of *Picoides* will be addressed in future work.

A third contradiction is that Short (1982) suggested that *Veniliornis* is distantly related to *Picoides,* and placed these genera in separate tribes. Short (1982) inferred that similarities between the two genera resulted from common ancestry of the Colaptini and Campetherini tribes. This study clearly shows a close sister relationship between *Veniliornis,* a South American genus, and South American *Picoides,* a relationship that follows predictions made by DeFilippis and Moore (2000). The relationships among all *Veniliornis* species and New World *Picoides* is currently under investigation. Finally, like *Veniliornis, Dendropicos* is more intimately related to *Picoides* than previously believed. The unresolved relationship between *Dendropicos* and Eurasian *Picoides* may be teased apart by including *P. obsoletus,* the African *Picoides,* but until appropriate collections are made, this question may remain unanswered.

Short used morphology, plumage characteristics, and behavioral traits to reconstruct phylogenetic relationships among several *Picoides* species. These types of data may be unreliable for phylogenetic analysis because they are often subject to selection leading to convergent evolution and homoplasy, and the genetic basis of defined morphological, behavioral, or plumage characters is typically not known (see Hillis, 1987; Avise, 1994). A DNA sequence-based phylogeny avoids these problems in phylogenetic analysis. Unfortunately, a well-resolved gene tree (Tateno *et al.,* 1982) may not accurately reflect the species tree because of lineage sorting (Nei, 1987; Pamilo and Nei, 1988; Wu, 1991). Lineage sorting is the process by which descendent species may acquire unique allele lineages from a pool of lineages carried by the common ancestor (i.e., alleles are polymorphic) (see Avise, 1994). The coalescence of these polymorphic alleles, the point in time of allelic divergence (Hudson, 1992), may not match the coalescence of species lineages. Thus a tree generated from a gene with longer coalescence branch lengths than the species tree will likely reflect a different arrangement of the species relationships than the true species tree (see Neigel and Avise, 1986). However, Moore (1995) showed that a mitochondrial haplotype tree has a high probability of correctly tracking species relationships because the small effective population size of the mitochondrial genome, resulting from haploidy and maternal inheritance, reduces the expected coalescence time. This study employed genetic sequences of the mitochondrial genome, the evolutionary properties of which are well understood, to reconstruct phylogenetic relationships, placing confidence that the mitochondrial gene tree is accurately tracking the species tree.

The crucial first step in studying the evolutionary history of groups of organisms is to have a correct phylogenetic framework (Felsenstein, 1985b; Omland 1999). Lester Short conducted extensive behavioral and ecological surveys of *Picoides* and other groups of woodpeckers that are invaluable to avian evolutionary biologists. However, his interpretation of these types of data in an evolutionary context is misleading due to the lack of a well-resolved phylogeny. Elucidation of several distinct groups that comprise the genus *Picoides* and paraphyly with *Veniliornis* and *Dendropicos* suggests that current systematic nomenclature does not accurately reflect evolutionary history and that the genus should be split. The mitochondrial gene tree presented here should help to clarify the systematics of the species now assigned to the genus *Picoides* and to foster future evolutionary studies of this large group of woodpeckers. Proposal of a systematic revision of these species is postponed until a parallel study based on nuclear gene intron sequences is completed.

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