

# A Test of a Mitochondrial Gene-Based Phylogeny of Woodpeckers (Genus *Picoides*) Using an Independent Nuclear Gene, $\beta$ -Fibrinogen Intron 7

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**A conservative estimate of the species tree for the woodpecker genus *Picoides* based on two mitochondrial protein-coding genes is tested using sequences of an independently evolving nuclear intron,  $\beta$ -fibrinogen intron 7. The mitochondrial gene-based topology and the intron-based topology are concordant, and a partition-homogeneity statistical test did not detect phylogenetic heterogeneity. The intron evolves more slowly than the mitochondrial sequences and tends not to resolve relationships among recently evolved species. However, the intron is superior over mitochondrial genes in resolving older bifurcations in the phylogeny. The two data sets were combined resulting in a robust estimate of the *Picoides* species tree in which most every node is statistically supported by bootstrap proportions. The *Picoides* species tree clearly shows that many morphological and behavioral characters used to lump species into this single genus have evolved by convergent evolution. *Picoides* is considered the largest genus of woodpeckers, but the molecular-based species tree suggests that *Picoides* is actually a conglomerate of several smaller groups.** © 2002 Elsevier Science (USA)

## INTRODUCTION

Reliance on a single gene tree to estimate the species tree can result in misinformed and incorrect interpretation of the evolutionary history of a group of species. The topology of an individual gene tree could differ from the species tree in the arrangement of species relationships (Ball *et al.*, 1990) if the gene tree possesses longer coalescence branch lengths than the species tree (Moore, 1995). An appreciable difference in coalescence branch lengths increases the probability of lineage sorting of polymorphic alleles (see Avise, 1994) making the true species tree difficult to identify (see Neigel and Avise, 1986). For this reason, it is important for systematic work to test rigorously molecular-based phylogenetic hypotheses using DNA sequences

from independently evolving linkage groups (Pamilo and Nei, 1988; Wu, 1991). Though individual gene trees generated from unlinked loci could differ in topology (Ball *et al.*, 1990), concordance among phylogenetic topologies based on sets of independently evolving data is good evidence that the true species tree has been recovered (Mickevich and Johnson, 1976; Mickevich, 1978; Penny and Hendy, 1986; Miyamoto and Fitch, 1995); discordance, on the other hand, provides insight into evolutionary process and population structure of groups of organisms (Lydeard *et al.*, 1995).

Weibel and Moore (2001) proposed a phylogenetic hypothesis of species relationships for the largest genus of woodpeckers (*Picoides*) (*sensu* Short, 1982) based on an aggregate DNA sequence data set of two mitochondrial protein-coding genes, cytochrome oxidase I (*COI*) and cytochrome b (*cyt b*) (see Discussion). Moore (1995) showed that a mitochondrial haplotype gene tree has a high probability of correctly tracking species relationships because the small effective population size of the mitochondrial genome, which results from haploidy and maternal inheritance, reduces the expected coalescence time. That is, the probability of lineage sorting of polymorphic alleles (Nei, 1987; Pamilo and Nei, 1988; Wu, 1991) is low in the mitochondrial genome.

The *Picoides* mitochondrial haplotype tree is likely devoid of spurious results in phylogenetic reconstruction (low random error) because it was generated from a large sample of nucleotide sites (Saitou and Nei, 1986; Nei, 1991). The high resolution of the tree is due to the desirable properties of mitochondrial DNA for phylogenetic analysis of recently evolved taxa (see Lanyon, 1988; Irwin *et al.*, 1991; see also Swofford *et al.*, 1996). Furthermore, most branch lengths of the *Picoides* phylogeny are sufficiently long to allow informative characters of rapidly evolving sequences to accumulate, and many nodes are well supported by bootstrap values, suggesting that saturation of mitochondrial sequences is not problematic (see Lanyon, 1988). However, *COI* and *cyt b* are inherited as a single

linkage group, and although each gene replicates the evolution of species relationships, the replicate data sets are not independent. Therefore, the mitochondrial DNA-based species phylogeny must be tested by generating a gene tree from sequences of an independently evolving nuclear gene. Because *Picoides* has evolved by a series of recent radiations resulting in short internodes in the mitochondrial gene tree (see Weibel and Moore, 2001), nuclear sequences used to resolve these nodes must have properties similar to those of the mitochondrial genome.

Nuclear intron sequences are fast becoming useful markers for deciphering phylogenetic relationships (e.g., Downie *et al.*, 1998; Oakley and Phillips, 1999; Walton *et al.*, 2000; Zhang, 2000). Introns evolve rapidly, and substitution rates are more or less uniform over the length of the sequence as they are typically not constrained by natural selection (but see Lewin, 1997). However, a clear difficulty with the use of introns, or other nuclear markers for that matter, is identifying orthologous sequences for phylogenetic analysis (Fitch, 1970). Thus, a good nuclear intron candidate should come from a single-copy gene in which the intron arrangement is conserved across a wide range of organisms. The size of the intron should be sufficiently large to reduce the probability of random error in phylogenetic analysis, and neutral substitutions should arise at a rate that is appropriate for resolving the relationships of species in question.

Prychitko and Moore (1997) developed primers for intron 7 of the  $\beta$  subunit of the fibrinogen gene ( $\beta$ -*fibint7*) and demonstrated the utility of  $\beta$ -*fibint7* in phylogenetic analysis of woodpeckers (Prychitko and Moore, 2000). Since its initial application,  $\beta$ -*fibint7* has been used to resolve species relationships in other avian systems (e.g., Johnson and Clayton, 2000).

The objective of this study is to test the mitochondrial DNA-based phylogenetic hypothesis of species relationships in *Picoides* woodpeckers (Weibel and Moore, 2001) by reconstructing the phylogeny using nuclear  $\beta$ -*fibint7* sequences. Concordance between the two gene trees suggests that the correct species tree has been recovered, and phylogenetic homogeneity among the three genes (*COI*, *cyt b*, and  $\beta$ -*fibint7*) permits combining the data sets to resolve virtually all nodes in the *Picoides* species tree.

## MATERIALS AND METHODS

Total DNA extracted from frozen or preserved tissues (Table 1) for a previous study (Weibel and Moore, 2001) was used for polymerase chain reaction (PCR) amplification of  $\beta$ -*fibint7* following the methods of Prychitko and Moore (1997). PCR products from two specimens, if available, of a given species were sequenced by automated sequencing. 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. Primers used for PCR and sequencing are listed in Table 2. Sequences were aligned by eye using ESEE (Cabot and Beckenbach, 1989) with published  $\beta$ -*fibint7* sequences for outgroups (see Table 1) to infer gap site locations of the intron resulting in 887 total sites (871 total sites for ingroup sequences alone). 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).

Methods of phylogenetic analysis followed those of Weibel and Moore (2001) and are summarized in Table 3. Briefly, maximum parsimony (MP) and neighbor-joining (NJ) (Saitou and Nei, 1987) analyses generated working topologies to serve as user-defined trees for a sequential optimization approach to maximum-likelihood (ML) analysis. The approach was modified from Frati *et al.* (1997) (see also Steppan *et al.*, 1999) to increase computational efficiency for large data sets. Four different substitution models were evaluated under a ML criterion. Six general time-reversible (GTR) rate matrix parameters, the proportion of invariable sites (I), and the gamma distribution shape parameter ( $\alpha$ ) for rate variation of nucleotides were simultaneously optimized under each substitution model and each working topology. A log likelihood test (see Goldman, 1993) determined the best substitution/rate variation model for explaining the data under each working topology. The statistically superior model(s) served as the optimized model(s) to heuristically search for the ML tree using tree bisection and reconnection (TBR) branch swapping and 10 random addition replicate data sets. All trees were rooted with a piculet (*Picumnus aurifrons*), a member of the sister subfamily (Picumninae) to the woodpecker subfamily (Picinae) (Short, 1982). Bootstrap analyses were performed on NJ and MP topologies with 1000 replicate data sets.

Sequences for the mitochondrial protein-coding genes *COI* (1551 of 1551 bases) and *cyt b* (1029 of 1143 bases) (Weibel and Moore, 2001) were combined with the  $\beta$ -*fibint7* sequences into a single data set of 3467 nucleotide sites. Phylogenetic analysis of the *COI*+*cyt b*+ $\beta$ -*fibint7* data set was performed according to the methods described above.

## RESULTS

With few exceptions,  $\beta$ -*fibint7* sequences were obtained for all specimens used in the Weibel and Moore (2001) molecular phylogenetic study of *Picoides*.  $\beta$ -*fibint7* could not be amplified from *Dendropicos griseocephalus* and two specimens of *P. borealis* (see Table 1 of Weibel and Moore, 2001).  $\beta$ -*fibint7* sequence was available for a different specimen of *S. varius* (Prychitko and Moore, 2000) than for *COI* and *cyt b* (DeFilippis and Moore, 2000 and

**TABLE 1**  
**List of Species (Order Piciformes, Family Picidae)**

Species	Common name <sup>b</sup>	Locale	Museum <sup>c</sup>	Voucher number	Intraspecific divergence <sup>d</sup>	Template sequence <sup>e</sup>
Subfamily Picinae						
Tribe Campetherini						
<i>Picoides albolarvatus</i>	White-headed WP	California, USA	WSU	86W-14.1	0	
<i>P. albolarvatus</i>	White-headed WP	California, USA	WSU	86W-14.5	—	X
<i>P. arcticus</i>	Black-backed WP	Montana, USA	WSU	86W-16.2	0.1	
<i>P. arcticus</i>	Black-backed WP	Montana, USA	WSU	86W-16.3	—	X
<i>P. borealis</i>	Red-cockaded WP	Florida, USA	FSU	209-1		X
<i>P. canicapillus</i>	Grey-capped WP	Primorskiy Kray, Russia	UW	51079		X
<i>P. kizuki</i>	Pygmy WP	Sakhalinskaya Oblast, Russia	UW	47374	0	
<i>P. kizuki</i>	Pygmy WP	Sakhalinskaya Oblast, Russia	UW	47379	—	X
<i>P. leucotos</i>	White-backed WP	Moscovskaya Oblast, Russia	UW	49580	0	
<i>P. leucotos</i>	White-backed WP	Moscovskaya Oblast, Russia	UW	49608	—	X
<i>P. lignarius</i>	Striped WP	Santa Cruz, Bolivia	LSU	6593		X
<i>P. maculatus</i>	Philippine WP	Philippines	USNM	607368		X
<i>P. major</i>	Great Spotted WP	Irkutskaya Oblast, Russia	UW	51700	0.3	X
<i>P. major</i>	Great Spotted WP	Krasnoyarskiy Kray, Russia	UW	51755	—	
<i>P. minor</i>	Lesser Spotted WP	Khabarovskiy Kray, Russia	UW	47225	0	X
<i>P. minor</i>	Lesser Spotted WP	Khabarovskiy Kray, Russia	UW	47226	—	
<i>P. mixtus</i>	Checkered WP	Provincia de Corrientes, Argentina	UW	810	0.1	X
<i>P. mixtus</i>	Checkered WP	Provincia de Corrientes, Argentina	UW	816	—	
<i>P. nuttallii</i>	Nuttall's WP	California, USA	WSU	86W-13.1	0	X
<i>P. nuttallii</i>	Nuttall's WP	California, USA	WSU	86W-13.3	—	
<i>P. pubescens</i>	Downy WP	Alabama, USA	WSU	86W-2.3	0	
<i>P. pubescens</i>	Downy WP	Texas, USA	WSU	86W-5.5	—	X
<i>P. scalaris</i>	Ladder-backed WP	New Mexico, USA	WSU	86W-8.2	0	X
<i>P. scalaris</i>	Ladder-backed WP	Arizona, USA	WSU	86W-11.7	—	
<i>P. stricklandi</i>	Strickland's WP	Arizona, USA	UA	16860		X
<i>P. tridactylus</i>	Three-toed WP	Sakhalinskaya Oblast, Russia	UW	47015	0.1	
<i>P. tridactylus</i>	Three-toed WP	Vologdaskaya Oblast, Russia	UW	49797	—	X
<i>P. villosus</i>	Hairy WP	Arizona, USA	WSU	86W-10.7	0.1	
<i>P. villosus</i>	Hairy WP	California, USA	WSU	86W-14.4	—	X
<i>Dendropicos fuscescens</i>	Cardinal WP	Kwa Zulu Natal Province, S. Africa	UW	471		X
Subfamily Picinae						
Tribe Colaptini						
<i>Veniliornis callonotus</i>	Scarlet-backed WP	Lambayeque, Peru	LSU	5178		
<i>V. nigriceps</i>	Bar-bellied WP	La Paz, Bolivia	LSU	8176		
<i>Colaptes auratus</i> <sup>a</sup>	Northern Flicker	Kentucky, USA	WSU	86-1.8		
<i>Piculus rubiginosus</i> <sup>a</sup>	Golden-olive WP	Lambayeque, Peru	LSU	5222		
Subfamily Picinae						
Tribe Melanerpini						
<i>Melanerpes carolinus</i> <sup>a</sup>	Red-bellied WP	Kentucky, USA	WSU	86W-1.4		
<i>Sphyrapicus varius</i> <sup>a</sup>	Yellow-bellied SS	Michigan, USA	WSU	85W-1.3		
Subfamily Picinae						
Tribe Campephilini						
<i>Dryocopus pileatus</i> <sup>a</sup>	Pileated WP	Texas, USA	WSU	86W-3.4		
Subfamily Picumninae						
Tribe Picumnini						
<i>Picumnus aurifrons</i> <sup>a</sup>	Bar-breasted Piculet	Santa Cruz, Bolivia	LSU	18254		

<sup>a</sup> Outgroup species;  $\beta$ -*fibint7* sequences (including *P. villosus*) were obtained from Prychitko and Moore (1997, 2000).

<sup>b</sup> WP, Woodpecker; SS, Sapsucker.

<sup>c</sup> FSU, Florida State University (F. James); LSU, Louisiana State University Museum of Natural Science; UA, University of Arizona; USNM, United States National Museum; UW, Burke Museum at University of Washington; WSU, Wayne State University (W.S. Moore).

<sup>d</sup> Percent sequence divergence between at least two conspecific taxa.

<sup>e</sup> A single specimen for a species serves as the template sequence.

Moore and DeFilippis, 1997, respectively). Prychitko and Moore (1997) provided *P. villosus*  $\beta$ -*fibint7* sequences, and their 2000 study provided  $\beta$ -*fibint7* sequences

for *V. callonotus* and *V. nigriceps*.  $\beta$ -*fibint7* sequences produced from this study are available from GenBank (accession numbers AF394307-AF394334).

TABLE 2

***β-fibint7* Primers for Amplification and Sequencing**

FIB7 primers	Sequence
FIB-B17U	5'-GGAGAAAACAGGACAATGACAATTCAC-3'
FIB-B17L	5'-TCCCCAGTAGTATCTGCCATTAGGGTT-3'
FIB-B17U2	5'-CATCCATGCAGTTCTGGCAATTCOAAGT-3'
FIB-B17L2	5'-TGGGAGGTGAAGCAGCTAAGAAAAACAA-3'

Note. Primers from Prychitko and Moore (1997).

Pairs of sequences obtained from two specimens of a given species were virtually identical, having at most 0.3% intraspecific sequence divergence (Table 1). All conspecific specimens paired as sister taxa in preliminary phylogenetic analysis indicating that contaminant DNA was not amplified and sequenced. 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 "synthetic" sequences following Weibel and Moore (2001). The best sequence of the two specimens served as the "template" sequence (Table 1). Missing data in the template sequence were filled using the homologous overlapping sequence of the second conspecific specimen. Homologous sites with different nucleotides across conspecific sequences were considered ambiguous and replaced with question marks for missing data in the synthetic sequence as these sites may differ due to sequencing error rather than real substitutions. The resultant alignment is available from Weibel (2001).

General time-reversible (GTR) distances (see Waddell and Steel, 1997) were used to generate a NJ tree from which the proportion of invariable sites (I) and the gamma distribution shape parameter for rate vari-

ation ( $\alpha$ ) were estimated using a ML criterion. A second NJ tree was then generated using GTR distances and the estimated parameters I and  $\alpha$ ; this second reconstruction served as the distance-based estimate of the *Picooides* woodpecker phylogeny and is shown in Fig. 1a.

Two MP analyses were performed using heuristic searches, TBR branch swapping, and 30 random addition replicate data sets. The first MP analysis used only *β-fibint7* sequence data and ignored all gaps. This search found 183 equally most-parsimonious trees; the strict consensus of all MP trees is shown in Fig. 1b. For the second MP analysis, a new character matrix was used in which gaps were coded and added to the sequence data set (see Prychitko and Moore, 2000). A gap was delimited to the greatest number of sites that would allow it to be coded unambiguously as an insertion (1) or a deletion (0) across all sequences. This method of coding is similar to that proposed by Lutzoni *et al.* (2000), except that they delimit the largest ambiguous region of an alignment as a single gap and code the variation of indels within the gap to reflect different states. The distinction is that here ambiguous regions of the aligned sequences are converted to many binary characters rather than fewer multistate characters, each representing independent evolutionary events. Twenty-three indels were interpreted from the manual alignment of *β-fibint7* sequences for all species used in this study. However, one indel (sites 437–441) was not included because the number of evolutionary events giving rise to its pattern could not be clearly and unambiguously interpreted (*sensu* Golenberg *et al.*, 1993). Of the 22 remaining indels (Table 4), only seven were synapomorphic; four indels (3, 4, 20, and 22) distinguished ingroup from outgroup species, two in-

TABLE 3

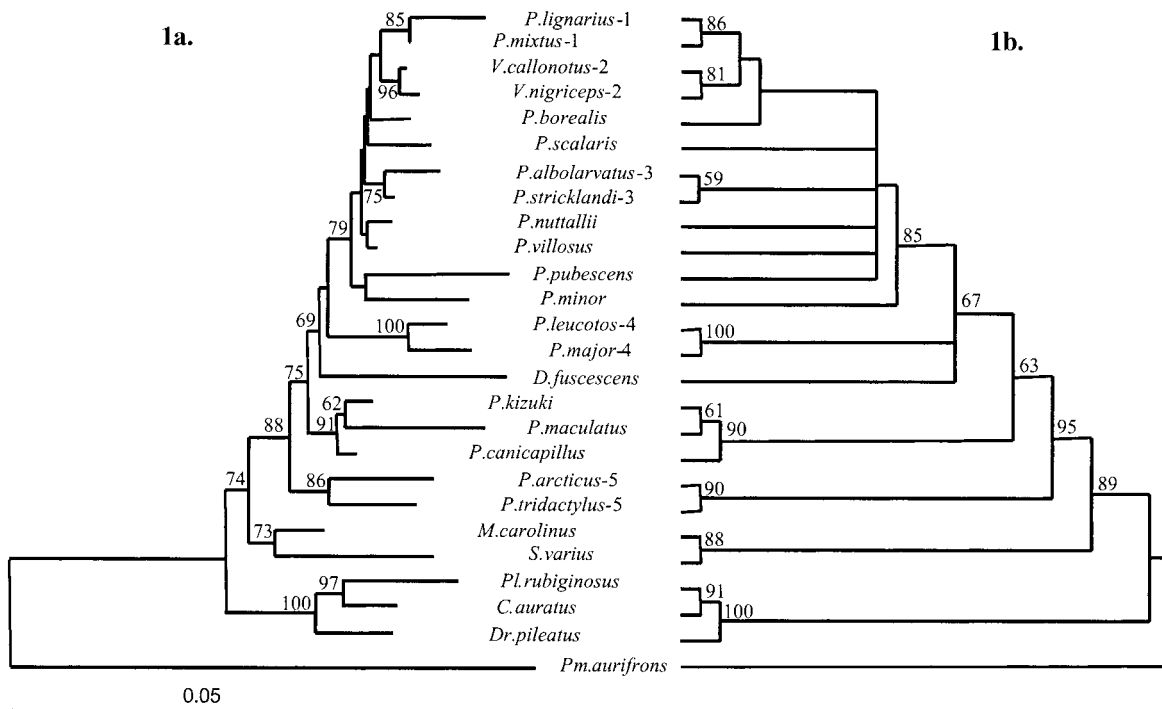
**Sequential Optimization for Maximum-Likelihood Phylogenetic Analysis**

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

<sup>a</sup> GTR rate matrix parameters, I, and  $\alpha$  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).

<sup>b</sup> A substitution/rate variation model = a substitution model + estimated rate parameters from step 2.

<sup>c</sup> A ML tree is generated from parameters estimated for the best substitution/rate variation model based on each working topology (NJ and MP); note that MP analysis may produce more than one working topology. Adopted from Weibel and Moore (2001).



**FIG. 1.** Phylogenetic reconstructions of *Picoides* with paraphyletic groups *Veniliornis* and *Dendropicos* generated from  $\beta$ -*fibint7* sequence data using the (a) neighbor-joining algorithm with GTR distances (minimum evolution score = 0.35133) and the (b) maximum parsimony criterion assuming equal weighting of characters. A strict consensus tree is shown (length=295, CI = 0.847, RI = 0.774, RC = 0.656). 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).

dels (6 and 10) provided limited information about the relationships among ingroup species, and one indel (14) united ingroup species and the *Melanerpes*–*Sphyrapicus* group. Inclusion of indels produced the same 183 MP topologies (data not shown) that were generated from  $\beta$ -*fibint7* sequences alone.

Neither the NJ topology (Fig. 1a) nor the MP topology (Fig. 1b) is well resolved at most nodes of recently diverged groups as indicated by bootstrap values of at least 70%, but resolution improves in deeper nodes. A bootstrap value of 70% based on a four-taxon simulation study is equated with a 95% probability that the node is real (Hillis and Bull, 1993) and is considered here as evidence of statistical support for nodes. The MP and NJ trees are subtly different; *P. minor* is sister to *P. pubescens* in the NJ tree but is sister to a clade of all New World species in the MP tree. Both analyses, however, clearly show that *Picoides* is paraphyletic with the South American *Veniliornis* and African *Dendropicos* genera and that Eurasian forms (*P. minor*, *P. leucotos*, *P. major*, *P. canicapillus*, *P. maculatus*, and *P. kizuki*) are interspersed among New World species of *Picoides*.

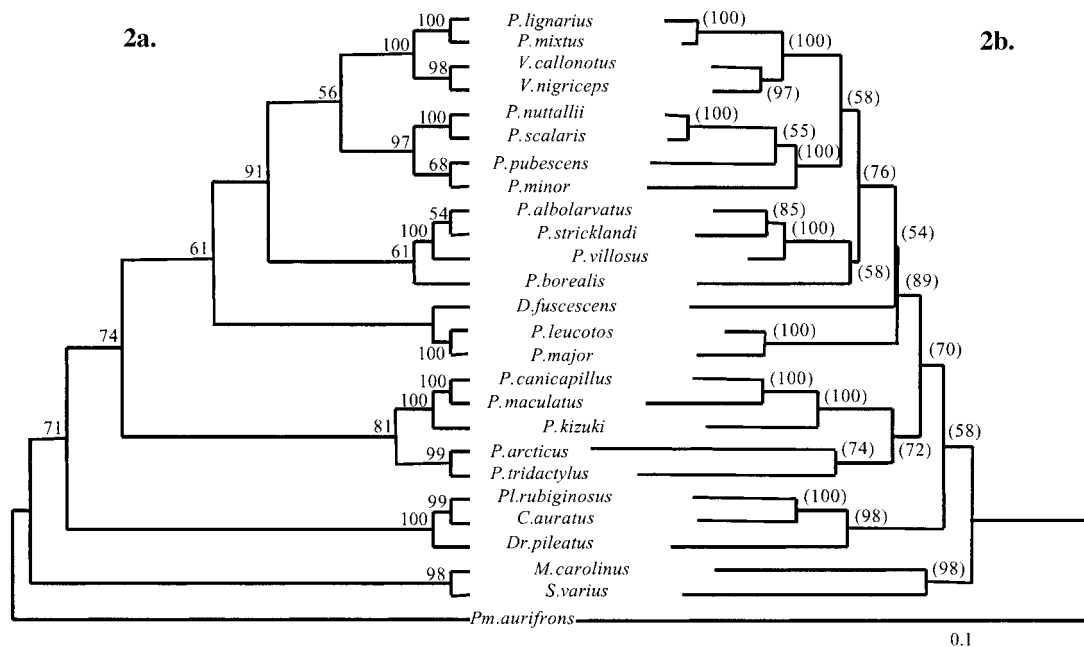
Because of the large number of MP trees produced from the  $\beta$ -*fibint7* sequence data and the lack of infor-

**TABLE 4**

**Indel Characters**

Indel number <sup>a</sup>	Nucleotide sites
1	18
2	19–22
3 (+)	25–29
4 (+)	30
5	31–32
6 (+)	33–34
7	98
8	129
9	163–165
10 (+)	247
11	425
12	433
13	498–505
14 (+)	565
15	664
16	728–739
17	746–749
18	790
19	803
20 (+)	845
21	846–851
22 (+)	852

<sup>a</sup> Indels are listed in 5'–3' direction of  $\beta$ -*fibint7* and (+) indicates that the indel is parsimony informative.



**FIG. 2.** Phylogenetic reconstructions of *Picoides* generated from the aggregate sequence data set (*COI*+*cyt b*+*β-fibint7*). Bootstrap values of at least 50% support for 1000 replicate data sets are shown at nodes. (a) Maximum-parsimony reconstruction assuming equal weighting of characters (length = 3730, CI = 0.443, RI = 0.452, RC = 0.200). (b) The resultant topology of a maximum-likelihood search using parameters for the GTR+I+ $\alpha$  model estimated from the neighbor-joining tree topology. Bootstrap values for the NJ topology (identical to the ML topology) are shown parenthetically at nodes.

mation gained by including indel data, an optimized ML model was not identified from either the sequence-based or sequence + indel-based MP analyses. The GTR+ $\alpha$  model was superior for explaining the *β-fibint7* data under the NJ working topology and parameter estimates for the GTR rate matrix and  $\alpha$  were used to search for the ML tree under this model. The resultant *β-fibint7* ML tree (not shown) is virtually identical to the NJ tree (Fig. 1a) except that, like the MP consensus tree (Fig. 1b), the ML tree places *P. minor* as the sister to the group that contains all New World species.

A partition-homogeneity test (Bull *et al.*, 1993) performed in PAUP\* under the criterion of maximum parsimony using heuristic search, TBR branch swapping, and 100 replicate data sets did not detect phylogenetic heterogeneity between the *COI*+*cyt b* and the *β-fibint7* data sets ( $P = 0.68$ ). The test determines whether variation among trees independently estimated from each data partition (in this case *COI*+*cyt b* vs *β-fibint7*) is significantly greater than the expected variation inherent within each estimate of phylogeny. Thus, the data sets were combined and phylogenetic analysis of the “three-gene” data set resulted in topologies with improved statistical support among nodes for recently and distantly diverged taxa.

Parsimony analysis of the three-gene data set generated a single MP tree (Fig. 2a). The GTR+I+ $\alpha$  model served as the optimal model for ML searches under both the MP and NJ working topologies for the three-

gene data set (see Table 3), and the resultant ML topologies generated from the NJ-based and the MP-based parameter sets are identical. The NJ working topology (not shown) is identical to the ML tree (Fig. 2b); the ML topology provides the best estimate of branch lengths, and statistical support for nodes on the ML topology is inferred from NJ bootstrap values. The three-gene MP topology (Fig. 2a) differs from the ML topology (Fig. 2b) in two areas. First, *P. minor* is the basal sister taxon to the “small” *Picoides* woodpeckers (*P. nuttallii*, *P. scalaris*, and *P. pubescens*) in the ML tree, whereas *P. minor* is sister to *P. pubescens* in the MP tree. Second, *D. fuscescens* is included in the Eurasian *P. leucotos*-*P. major* clade in the MP tree, whereas ML places *D. fuscescens* as the sister taxon to the group of New World *Picoides* including *P. minor*. None of these relationships is statistically supported.

## DISCUSSION

A strict consensus tree of the NJ, MP, and ML topologies based on *COI*+*cyt b* DNA sequence data (Fig. 2b in Weibel and Moore, 2001) summarizes the results of the three analyses by collapsing nodes that are ambiguous (de Queiroz, 1993). This phylogeny thus serves as a conservative and best estimate of the *Picoides* species tree without inferring relationships that are actually weakly supported. In general, the mitochondrial DNA-based tree shows that the New World species form

three distinct groups: the “large” North American species (*P. albolarvatus*, *P. villosus*, *P. stricklandi*, and *P. borealis*), the “small” North American species (*P. nuttallii*, *P. scalaris*, and *P. pubescens*) including *P. minor* (a Eurasian species), and a South American group that includes *P. lignarius*, *P. mixtus*, and representatives of the genus *Veniliornis*. The relationships among these three groups are not clear. The African genus *Dendropicos* and the Eurasian species (*P. leucotos* and *P. major*) are clearly closely related to the New World group, and the remaining Eurasian species used in this study and the North American “three-toed” woodpeckers (*P. arcticus* and *P. tridactylus*—*P. tridactylus* has a holarctic distribution) form a distinct group.

Although  $\beta$ -*fibint7* NJ (Fig. 1a) and MP (Fig. 1b) trees do not have the same level of resolution as the *COI+cyt b* tree,  $\beta$ -*fibint7* trees arrange taxa into groups that are overall in concordance with the *COI+cyt b* phylogeny.  $\beta$ -*fibint7* unites the New World group (including *P. minor*) into a distinct group, maintains the paraphyletic relationship between South American *Picoides* and *Veniliornis* species, and affirms paraphyly of *Picoides* with *Dendropicos*. Minor discordance between the *COI+cyt b* tree and  $\beta$ -*fibint7* trees occurs at deeper splits. *COI+cyt b* arranges the Eurasian species (*P. canicapillus*, *P. maculatus*, and *P. kizuki*) and the “three-toed” woodpeckers into a distinct group, whereas  $\beta$ -*fibint7* trees place the Eurasian species and the “three-toed” species into separate lineages. Arrangement of outgroup species is similar among both data sets, but  $\beta$ -*fibint7* places the *Melanerpes-Sphyrapicus* group as sister to all *Picoides*, whereas the *Piculus-Colaptes-Dryocopus* group appears to be more closely related to *Picoides* in the *COI+cyt b* tree. Interestingly, Pritchitko and Moore (2000) found the same patterns of relationships between *Picoides* and these outgroup species even though they included only a single representative of *Picoides* (*P. villosus*) in their analysis. The apparent sister relationship between *Picoides* and the *Melanerpes-Sphyrapicus* group recovered by the  $\beta$ -*fibint7* data may be driven in part by an insertion (indel 14, Table 4) that is held in common by all ingroup species (Table 1) and the *Melanerpes-Sphyrapicus* group.

The *COI+cyt b* and  $\beta$ -*fibint7* data sets serve as replicates of the evolutionary history (see Pritchitko and Moore, 1997) of *Picoides*. Overall, there is phylogenetic concordance among trees generated from both sets of independently evolving DNA sequences. Thus, the species tree inferred from the *COI+cyt b* gene tree is generally supported by  $\beta$ -*fibint7* sequence data.

Relative substitution rates and patterns of nucleotide substitution were compared between the *COI+cyt b* and  $\beta$ -*fibint7* data sets. Pritchitko and Moore (2000) made similar comparisons between *cyt b* and  $\beta$ -*fibint7* for several distantly related species. In this study, a larger sample of recently diverged species provides a

better estimate of evolutionary rates and substitution patterns than those generated by the Pritchitko and Moore (2000) study. Branch lengths of the three-gene phylogeny were estimated by ML (Yang, 1994) in PAUP\* using *COI+cyt b* and  $\beta$ -*fibint7* sequence data, respectively. The slope of the regression of *COI+cyt b* branch lengths on  $\beta$ -*fibint7* branch lengths was significantly different from zero ( $P < 0.001$ ; slope = 3.971; 95% confidence interval: 3.839–4.103). The slope indicates that the mitochondrial genes evolve 3.971 times as fast as  $\beta$ -*fibint7*. This is a higher, and presumably better, estimate than that made by Pritchitko and Moore (2000) because this estimate is based on more closely related species and is less affected by multiple substitutions. Table 6 shows estimates for substitution rate ratios for the R matrix and the gamma shape parameter,  $\alpha$  (Yang, 1996), estimated by maximum-likelihood (Yang, 1994) for the *COI+cyt b* and  $\beta$ -*fibint7* data sets using the three-gene ML tree (Fig. 2b). Rate ratios are proportional to the conditional probabilities that a given nucleotide (A, C, G, or T) will be substituted by another nucleotide over a small increment of time. The  $\alpha$  shape parameter describes the continuous distribution of substitution rates among nucleotide sites partitioned over three discrete categories. Following Pritchitko and Moore (2000), rate ratios were compared between the two data sets. The R-matrix elements of the *COI+cyt b* data set were transformed to the same scale as the  $\beta$ -*fibint7* R-matrix elements by multiplying the *COI+cyt b* rate ratios by 3.971, the multiple by which the *COI+cyt b* substitution rate exceeds the  $\beta$ -*fibint7* substitution rate (Pritchitko and Moore (2000) transformed the *cyt b* R-matrix elements in the same manner as described here, but an error in wording in their paper indicates that the R-matrix elements for the  $\beta$ -*fibint7* data set, not the *cyt b* data set, were multiplied by the slope of the regression of branch lengths).

The two data sets exhibit important differences that are indicative of their ability to resolve different regions of the *Picoides* phylogeny. The *COI+cyt b* data set is nearly three times larger than the  $\beta$ -*fibint7* data set and has a higher percentage of variable sites (Table 5).  $\beta$ -*fibint7* sequences are A-T rich (see also Pritchitko and Moore, 1997) relative to the mitochondrial sequences (Table 5), a phenomenon that reflects higher frequency of mutation of C and G than A and T in noncoding portions of a gene with most mutations resulting in A or T (Li and Graur, 1991). Overall,  $\beta$ -*fibint7* sequences evolve at slower rates than *COI+cyt b* sequences and with greater uniformity across all types of transitions and transversions, whereas *COI+cyt b* sequences undergo transitional substitutions that far exceed moderately variable transversional changes (see also Table 5). The substantial rate variation among sites in the mitochondrial sequence data set is reflected by a low estimate of the

**TABLE 5**  
**Analysis of  $\beta$ -*fibint7* Sequence Data for Ingroup Woodpeckers**

	<i>COI+ cyt b</i> <sup>b</sup>	$\beta$ - <i>fibint7</i> <sup>c</sup>																	
Total nucleotide sites	2580 bases	871 sites																	
Variable sites	910 sites	139 sites																	
Transition:transversion <sup>a</sup>	10.4 (ML estimate = 6.7)	0.82 (ML estimate = 0.83)																	
Variable sites of total sites	35.3%	16.0%																	
Mean % nucleotide composition ± 1 standard deviation	<table border="0"> <tr> <td rowspan="4" style="font-size: 3em; vertical-align: middle;">{</td> <td>A</td> <td>25.5 ± 0.35</td> </tr> <tr> <td>C</td> <td>33.8 ± 0.56</td> </tr> <tr> <td>G</td> <td>15.2 ± 0.31</td> </tr> <tr> <td>T</td> <td>25.5 ± 0.57</td> </tr> </table>	{	A	25.5 ± 0.35	C	33.8 ± 0.56	G	15.2 ± 0.31	T	25.5 ± 0.57	<table border="0"> <tr> <td>A</td> <td>31.1 ± 0.37</td> </tr> <tr> <td>C</td> <td>17.2 ± 0.41</td> </tr> <tr> <td>G</td> <td>18.0 ± 0.27</td> </tr> <tr> <td>T</td> <td>33.8 ± 0.44</td> </tr> </table>	A	31.1 ± 0.37	C	17.2 ± 0.41	G	18.0 ± 0.27	T	33.8 ± 0.44
{	A		25.5 ± 0.35																
	C		33.8 ± 0.56																
	G		15.2 ± 0.31																
	T	25.5 ± 0.57																	
A	31.1 ± 0.37																		
C	17.2 ± 0.41																		
G	18.0 ± 0.27																		
T	33.8 ± 0.44																		

<sup>a</sup> Ratio was analyzed by using independent pairs of recently diverged taxa (see numerically paired species in Fig. 1), and values in parentheses were estimated by maximum-likelihood.

<sup>b</sup> Data were extracted from the aggregated *COI+ cyt b* data set of joined sequences.

<sup>c</sup> Gap sites imposed by alignment with outgroup species are eliminated.

$\alpha$  shape parameter; the larger estimate of  $\alpha$  for the  $\beta$ -*fibint7* data set suggests less rate variation among nucleotide sites (see Yang, 1996).

$\beta$ -*fibint7* sequences, like mitochondrial gene sequences, have properties that are ideal for phylogenetic analysis (Prychitko and Moore, 1997, 2000): a sufficient number of nucleotide sites that evolve under neutral evolution such that substitutions follow a Poisson distribution and a relatively high substitution rate. Thus  $\beta$ -*fibint7* should resolve interspecific relationships of recently diverged species comparable to the mitochondrial genes (see Prychitko and Moore, 2000). However,  $\beta$ -*fibint7* evolves more slowly than the mitochondrial genes (Tables 5 and 6) (see also Prychitko

and Moore, 2000) which explains why the intron is superior for resolving older splits but cannot resolve recent splits among *Picooides* species as well as the mitochondrial genes. This pattern is also inferred from phylogenetic analysis of pigeon and dove *cyt b* and  $\beta$ -*fibint7* sequences (Johnson and Clayton, 2000). The utility of  $\beta$ -*fibint7* in resolving relationships among recently evolved species is limited relative to mitochondrial DNA sequences because of the smaller number of available nucleotide sites and slower substitution rate. However, the intron retains substantial phylogenetic information that is useful for resolving the deeper relationships among *Picooides* species that are beyond the resolving power of the mitochondrial genes. Thus, the mitochondrial genes (*COI* and *cyt b*) and the nuclear intron ( $\beta$ -*fibint7*) offer different windows of resolution for phylogenetic tree reconstruction.

The debate of whether to combine independent data sets for phylogenetic analysis is ongoing and arguments fall into two theoretical camps. The first is that of taxonomic congruence in which species phylogenies are inferred from agreement among supported topologies generated from different data sets (i.e., data should not be combined) (Mickey, 1978). Arguments in favor of taxonomic congruence suggest that independent data sets should not be combined if they strongly support conflicting trees. The second camp promotes character congruence in which combining individual data sets maximizes descriptive efficiency and explanatory power, and the true species phylogeny is inferred from the total available evidence (Kluge, 1989; Eernisse and Kluge, 1993). It is argued that combining data sets resolves character conflicts, and there is no burden of identifying natural partitions in the data that may have different evolutionary histories. Proponents of character congruence further argue that it is better to combine data prior to phylogenetic analysis because different methods of constructing consensus trees from independently analyzed data sets may result in different topologies and may not necessarily be

**TABLE 6**

**Among-Site Rate Variation Parameter Estimates for  $\beta$ -*fibint7* and *COI+ cyt b* Sequence Data**

$\beta$ - <i>fibint7</i>		A	C	G	T
R matrix:					
	A	—	2.165	1.953	0.488
	C		—	1.662	2.902
	G			—	1.000
	T				—
$\alpha = 0.697$					
<i>COI+ cyt b</i>		A	C	G	T
R matrix:					
	A	—	3.083	39.232	3.898
	C		—	1.968	38.015
	G			—	3.971
	T				—
$\alpha = 0.193$					

*Note.* All sites in *COI+ cyt b* (first-, second-, and third-codon positions) and  $\beta$ -*fibint7* data sets were gamma corrected for rate variation among sites for three categories (Yang, 1996). Rate ratios of the *COI+ cyt b* R matrix were transformed to the  $\beta$ -*fibint7* scale by multiplying its elements by 3.971, the multiple by which the substitution rate of the mitochondrial genes exceeds the substitution rate of  $\beta$ -*fibint7* (see text).

the most conservative estimates of the species tree. An excellent review of the taxonomic congruence–character congruence debate is presented by Miyamoto and Fitch (1995) (see also Huelsenbeck *et al.*, 1996). Huelsenbeck *et al.* (1996) offer a third choice regarding multiple data sets and phylogenetic analysis, conditional data combination. Independent data sets could be combined if they are not significantly heterogeneous. Data should not be combined if they produce phylogenies that are statistically incongruent. Thus, phylogenetic analysis should be performed initially on independent data sets to identify discordance among topologies; if there is no phylogenetic heterogeneity, then data should be combined and reanalyzed phylogenetically.

The *COI+cyt b* and  $\beta$ -*fibint7* data sets were initially analyzed independently, and the resultant topologies are in statistical agreement at deeper nodes for the same general groups of taxa even though recent interspecific relationships are not resolved by  $\beta$ -*fibint7*. There is no statistical evidence from a partition-homogeneity test that the data are partitioned; *COI+cyt b* and  $\beta$ -*fibint7* are phylogenetically homogeneous (see Bull *et al.*, 1993) and should be combined into a single data set for phylogenetic analysis (Huelsenbeck *et al.*, 1996). The two data sets are evolving at different rates and interact positively to resolve relationships in different areas of the phylogenetic tree (Hillis, 1987). Though these data sets may support different models of evolution due to differences in transition to transversion ratios, base compositional biases, or substitution rates, the topologies reconstructed from these data sets independently are essentially identical. Log likelihood ratio tests (see Goldman, 1993) show improvement in the explanatory power of different models of evolution for mitochondrial, nuclear, and the three-gene data sets, but the ultimate answer (i.e., the estimation of the *Picoides* species tree) is consistent among the data sets.

Three methods of phylogenetic analysis (MP, NJ, and ML) performed on the three-gene data set produced two virtually identical topologies. The conflicts between the MP topology (Fig. 2a) and the ML/NJ topology (Fig. 2b) occur at very short internodes that are not statistically supported by bootstrap values. The GTR+I+ $\alpha$  model used in this study to optimize the ML search has the shortcoming of increased variance in estimation of topologies or branch lengths due to the sheer number of parameters that are estimated, but the model offers the highest explanatory power over other evolutionary models because it is parameter rich. A benefit of the ML method is that it tends to be less sensitive to violations in model assumptions (Felsenstein, 1981, 1988; Huelsenbeck, 1995a, 1995b), such as unequal or high variance in parameter estimation, and branch lengths can be more reliably estimated (see Felsenstein 1981, 1988; see also Huelsenbeck, 1995b

and Swofford *et al.*, 1996). Therefore, the ML tree (Fig. 2b) is considered “the best” estimate of the *Picoides* species tree given the available taxa for this study. Forthcoming research on character evolution, biogeography, and taxonomy of *Picoides* woodpeckers will be based on the ML *Picoides* species tree presented here.

It is clear that the genus *Picoides* as presently defined (see Delacour, 1951; Short, 1971, 1982) is an arbitrary collection of species that were lumped together based on morphological and behavioral characters. The molecular-based species tree presented here has been tested by two independent DNA sequence data sets and shows that similarities in nonmolecular characters arose via convergent evolution. *Picoides* should not be considered the largest genus of woodpeckers but rather a conglomerate of several smaller groups; whether those “groups” represent true genera will be determined in future work.

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