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THE USE OF CYTOCHROME *B* SEQUENCE VARIATION IN ESTIMATION OF PHYLOGENY IN THE VIREONIDAE[1]

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Abstract. A recent phylogenetic study of the genus *Vireo*, based on protein electrophoresis, shows the taxon is polyphyletic and contains four distinct groups. This contradicts the traditional classification of the genus. Johnson et al. (1988) find a group containing members of the subgenera *Vireo* and *Lanivireo*, a lone taxon containing the species *V. bellii* and a division of the subgenus *Vireosylva* into a *gilvus* and an *olivaceus* group. To test these results independently, sequence data from the mitochondrial cytochrome *b* (cyt-*b*) gene were collected from the following 9 vireos representing the three subgenera: Bell's Vireo (*Vireo bellii*), Gray Vireo (*V. vicinior*), Solitary Vireo (*V. solitarius*), Black-capped Vireo (*V. atricapillus*), White-eyed Vireo (*V. griseus*), Philadelphia Vireo (*V. philadelphicus*), Warbling Vireo (*V. gilvus*), Red-eyed Vireo (*V. olivaceus*) and Black-whiskered Vireo (*V. altiloquus*), and two confamilial species: Rufous-browed Peppershrike (*Cyclarhis gujanensis*) and Slaty-capped Shrike-Vireo (*Vireolanius leucotis*). For each of the above species, at least 273 homologous base pairs from the cyt-*b* gene were amplified using the polymerase chain reaction, and subsequently sequenced. Estimates of phylogenies were achieved through phenetic, maximum likelihood and weighted cladistic analyses. The evolutionary histories produced did not support or reject the monophyly of the genus *Vireo*. However, *Cyclarhis* was more closely related to *Vireo* than was *Vireolanius*. Two major clades were found in the genus *Vireo*. An eye-lined clade contained members of the subgenus *Vireosylva* while an eye-ringed group contained members of the subgenera *Vireo* and *Lanivireo* (including *V. bellii*).

Key words: *Cytochrome* b; Vireo; Cyclarhis; Vireolanius; *phylogeny; mitochondrial DNA.*

INTRODUCTION

The New World passerine family Vireonidae is comprised of four genera; *Vireo, Hylophilus, Vireolanius* and *Cyclarhis. Vireo* is the largest of these genera, containing 26 species (AOU 1983), 11 of which occur in North America. Based on a variety of morphological and behavioral characters, the genus *Vireo* is currently subdivided into three subgenera (*Vireo, Lanivireo* and *Vireosylva*) by the American Ornithologists' Union (1983). However, validity of the subgenus *Lanivireo* is doubtful, with most authors placing its members within the subgenus *Vireo* (Barlow 1980 and unpublished allozyme data, Johnson et al. 1988). Thus, there are only two major clades in the genus, which are commonly termed the eye-ringed vireos (subgenus *Vireo* including *Lanivireo*) and the eye-lined vireos (subgenus *Vireosylva*).

Species within the genus show a high amount of molecular divergence relative to other passerine congeners (Avise et al. 1982, Johnson et al. 1988), which belies some morphological and be-

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havioral similarities (Barlow and James 1975, Barlow 1980, Barlow and Nash 1985). Johnson et al. (1988) reported a mean genetic distance (Nei 1978) of 0.29 within *Vireo.* This value is small when compared to nonavian vertebrates, but is three times larger than the mean genetic distance among the species of the genus *Anas* and at least four times larger than mean distances among species within the passerine genera, *Melospiza, Dendroica* and *Parus,* studied to date (see Tegelstrom and Gelter 1990, for a review of genetic distances). This large distance may be due to two factors which are not mutually exclusive. The taxon may be relatively old (Avise et al. 1982) or it may be undersplit at the generic and specific levels (Johnson et al. 1988).

Johnson et al. (1988), in an extensive electrophoretic survey of the family Vireonidae that included a number of the commonly recognized species, found four main taxonomic groupings within the genus *Vireo*: an eye-ringed group (*V. griseus, V. solitarius, V. flavifrons, V. vicinior, V. huttoni* and *V. carmioli*), the seemingly distinctive *V. bellii* (previously grouped with eye-ringed taxa), and two eye-lined groups, the *gilvus* group (*V. gilvus* and *V. philadelphicus*) and the *olivaceus* group (*V. olivaceus* and *V. altioquus* in this study). Additionally, the confamilial genera *Vireolanius, Cyclarhis* and *Hylophilus* clustered with members of the genus *Vireo,* suggesting that *Vireo* is a polyphyletic taxon. Also from these data, Johnson et al. suggested that the four abovementioned taxonomic groupings be raised to generic rank. They declined, however, to make this a formal recommendation. They do recommend, formally, the disuse of the categories of subgenera within *Vireo.*

MOLECULAR ANALYSIS

The appropriate region of DNA to consider in a phylogenetic study is a function of the relatedness of the taxa examined. Mitochondrial DNA (mtDNA) is ideal for phylogenetic analysis of closely-related taxa because it evolves about 10 times faster on average than nuclear DNA (Brown 1985). In addition, it is a haploid, maternally-inherited, genome which makes it easier to characterize than diploid, nuclear DNA. In the class Aves, mitochondrial DNA variation has been used to examine species relationships within genera and within species, e.g., waterfowl (*Anas*), sparrows (*Melospiza*), warblers (*Dendroica*) (Kessler and Avise 1985), geese (*Anser,* Shields and Wilson 1987; *Branta,* Quinn et al. 1991), old world leaf warblers (*Phylloscopus,* Richman and Price 1992), chickadees and titmice (*Parus,* Mack et al. 1986; Gill et al. 1993), sparrows (*Ammodramus,* Zink and Avise 1990; *Zonotricha,* Zink et al. 1991; *Amphispiza,* Johnson and Cicero 1991), blackbirds (Icterinae, Lanyon 1992), flycatchers (*Ficedula,* Tegelstrom and Gelter 1990), francolins (*Francolinus,* Crowe et al. 1992), Australian rosellas (*Platycercus,* Ovenden et al. 1987) and Australian babblers (*Pomatostomus,* Edwards and Wilson 1990).

In these studies, except those of Edwards and Wilson (1990), Johnson and Cicero (1991), Richman and Price (1992) and Lanyon (1992), mitochondrial variation was characterized through restriction fragment analysis. In the latter papers, cytochrome *b* sequence variation was used to estimate genetic variation. Cytochrome *b* is essential for the proper transfer of electrons in the electron transfer chain. It is one of the 9–10 proteins that combine to form complex III of the mitochondrial oxidative phosphorylation system (Hatefi 1985). This complex transfers electrons from dihydroubiquinone to cytochrome *c* which is coupled with the translocation of proteins across the inner mitochondrial membrane (Hatefi 1985). Cytochrome *b,* a membrane bound protein, is one of the most conserved regions of the mitochondrial DNA (Desjardins and Morais 1990).

The complete sequence and mitochondrial position of cyt-*b* in birds has been characterized in chickens (Desjardins and Morais 1990). It is a protein of 380 amino acids in length, located not adjacent to the D-loop, as in mammals, but is separated from it by the ND6 gene.

As an independent test of the results of Johnson et al. (1988) sequence data from a portion of the mitochondrial cytochrome *b* (91 amino acids) gene were used to reconstruct an estimate of the phylogenetic history of the genus. Independently derived phylogenies were compared directly.

MATERIALS AND METHODS

SAMPLE COLLECTION

DNA was isolated from tissue samples representing nine *Vireo* species commonly found in North America, for two species of the confamilial genera *Vireolanius* and *Cyclarhis* and the Black-billed Magpie (*Pica pica*). The Black-billed Mag-

pie was used as an outgroup, based in part on the recommendation of Sibley and Ahlquist (1982, 1990) that vireos and magpies (Corvidae) are in the same parvorder Corvidea. For each sample, liver, heart, and pectoral muscle were either frozen on solid CO_2 or in liquid N_2 following collection and stored in a −70°C freezer. All samples were provided by either the Provincial Museum of Alberta, Edmonton; the Royal Ontario Museum, Toronto; Louisiana State University, Baton Rouge; or the Field Museum of Natural History, Chicago. The species collected, sample sizes, geographic location of the acquisitions, and corresponding haplotypes are listed in Table 1.

AMPLIFICATION OF CYTOCHROME *b*

One to 2 ml of sample representing about 100 ng of DNA was used to start the reaction. The reaction mixture contained 10 ml of a 10× standard PCR buffer (2.0 mM $MgCl_2$ final concentration), 200 nM dNTPs, 1 unit of Taq DNA polymerase (Promega), 20 pmol of each primer, was made up to a volume of 100 ml with deionized H_2O and covered by a layer of oil to prevent condensation. The primers were H15149 (5′ > CCTCA GAATG ATATT TGTCC TCA < 3′) and a modified version of L14841 (5′ > ATCCA ACATC TCAGC ATGAT GAAA < 3′) as described by Kocher et al. (1989). The modification involved the removal of five to six base pairs at the 5′ end. The program for the thermal cycler was as follows: 93°C for 5 min, 54°C for 1 min and 72°C for 2 min. This initial cycle was followed by a slightly different cycle which was repeated 30 times. This cycle was 94°C for 15 sec, 54°C for 30 sec, and 72°C for 2 min. The final cycle was the same as the latter except the reaction was held by 72°C for 10 min.

ISOLATION OF PCR PRODUCT

To ensure that the desired PCR product was sequenced, the product was excised from a 1% low melting point (LMP) agarose gel. The LMP wedge was dissolved in deionized H_2O up to a volume of 1 ml by heating for 10 min at 65°C (Gyllensten 1989). This solution was then used as the template for asymmetric amplification of the product. For each sample two separately produced products were obtained. For the cytochrome *b* amplifications, only one product was visualized on the gels.

ASYMMETRIC AMPLIFICATION

Production of single stranded DNA was facilitated by limiting one of the primers to a low concentration. The reaction concentrations were as follows: 40 pmol of one primer, 0.5 pmol of the other primer, 100 nM dNTPs, 10 μl of a 10× asymmetric reaction buffer (0.5 mM $MgCl_2$ final concentration), 2 units of Taq polymerase, H_2O up to a final volume of 100 μl and covered by 75 μul of mineral oil. The cycles for the asymmetric reaction were identical to the initial PCR except the middle amplification cycles were repeated 40 times.

For every sample, one of the two isolated products was used as a template to produce a specific single stranded DNA while the other product was used as a template to produce the other complementary strand. In this way, any errors of the PCR reaction could be identified during sequencing.

SEQUENCING OF THE ASYMMETRIC PRODUCT

The US Biochemical Sequenase kit Version 2.0 and the Pharmacia^{T7} sequencing kit were both used to sequence the single stranded product. The only deviation made from the recommended protocols was that no elongation step was carried out.

ESTIMATES OF PHYLOGENY

Phenetic analyses. Two separate estimates of phylogeny were derived from the sequence variation seen in Table 2 with the aid of the computer package Phylip 3.5c (Felsenstein 1993). The program DNAdist was used to calculate corrected DNA distance values based on Kimura's "two-parameter" model (1980). A 2-to-1 rate of transition to transversion substitution was used to create the values seen in the upper diagonal of Table 3. The tree in Figure 3 was constructed using the Neighbor program included in the Phylip package. Bootstrap analysis of this tree was performed with 1,000 replicates, using this tree building method. Figure 4 was produced through the Fitch-Margoliash and least square methods employed by the Fitch program with the global rearrangement option. Bootstrap analysis of the Fitch tree was again performed using the same Fitch options on 1,000 replicates.

In both trees, the confidence values of the internal lineages were assessed by a bootstrap analysis (Felsenstein 1985) using the options avail-

TABLE 1. Sample location, sample size and cyt-*b* haplotypes of the vireonids studied and the outgroup *Pica pica*.

Taxa	Sample collection location	*n*	Haplotypes
Family Vireonidae			
Subfamily Vireoninae			
Genus *Vireo*			
Eye-lined vireos			
Subgenus *Vireosylva*			
Warbling Vireo			
Vireo gilvus gilvus	North Battleford, Saskatchewan	2	Vgg B, Vgg C
	Beaverhills Lake, Alberta	3	Vgg A (2), * Vgs
Vireo g. swainsonii	Castle River, Alberta	2	Vgs (2)
	Hailstone Butte, Alberta	2	Vgs (2)
	Cypress Hills, Alberta	1	Vgs
Philadelphia Vireo			
Vireo philadelphicus	Weyakwin, Saskatchewan	1	Vph
Black-whiskered Vireo			
Vireo a. altiloquus	Jamaica	1	Val
Red-eyed Vireo			
Vireo olivaceus	Weyakwin, Saskatchewan	1	Vol A
	Winefred Lake, Alberta	2	Vol B (2)
Eye-ringed vireos			
Subgenus *Vireo*			
Bell's Vireo			
Vireo bellii bellii	Tom Green County, Texas	1	Vbe A
	Cleveland County, Oklahoma	1	Vbe B
White-eyed Vireo			
Vireo griseus	Louisiana	1	Vgr
Black-capped Vireo			
Vireo atricapillus	Kerr County, Texas	1	Vat
Subgenus *Lanivireo*			
Gray Vireo			
Vireo vicinior	Brewster County, Texas	1	Vvi
Solitary Vireo			
Vireo solitarius solitarius	Jackfish Lake, Saskatchewan	1	Vss A
	Seven Mile Creek, Alberta	1	Vss B
	Nordegg, Alberta	1	Vss B
	Clearwater River, Alberta	1	* Vsc
Vireo s. cassinii	Windsor Ridge, Alberta	3	Vsc (3)
	Hailstone Butte, Alberta	1	Vsc
	Seven Mile Creek, Alberta	1	Vsc
Subfamily Cyclarhinae			
Genus *Cyclarhis*			
Rufous-browed Peppershrike			
Cyclarhis gujanensis	Venezuela	1	C.gu
Subfamily Vireolaninae			
Genus *Vireolanius*			
Slaty-capped Shrike-vireo			
Vireolanius leucotis	Venezuela	1	Vl.le
Family Corvidae			
Black-billed Magpie			
Pica pica	Edmonton, Alberta	1	P.pi
	Totals	$n = 32$	Haplotypes = 20

An '*' indicates the occurrence of a haplotype most commonly associated with the other subspecies. Bracketed numbers indicate number of samples with the given haplotype at each location. Haplotype sequence data are found in Table 3 (for example, Vgg b = V.g.gilvus B).

able in the programs. In each analysis, 1,000 bootstrap data bases were created from which trees were constructed. A consensus tree of the bootstrap trees was made with the program Consensus, which constructed a majority rule tree. This program produces a consensus tree that consists of all groups that occur most often in the replicates. Although it is a majority rule tree, branch points found less than 50% of the time will be shown if contradictory branches of equal value do not exist. Ninety-five of 100 trees possessing a given branch point was considered as a significant level of confidence for that branch point.

Maximum likelihood estimate. The tree in Figure 5 was constructed with the DNAml program in the Phylip package. This program examines all sites in a sequence and assumes all sites and lineages evolve independently and that each site undergoes a substitution at a given rate. As in the phenetic analyses a 2-to-1 transition to transversion substitution ratio was set and the global rearrangement option was invoked.

Cladistic analyses. All cladistic analyses were performed with the computer programs PAUP 3.0 (g) (Swofford 1989) and MacClade 2.1 (Maddison and Maddison 1987). The most parsimonious trees found by PAUP using unordered, fully reversible characters (Fitch parsimony) were further analyzed by MacClade to gain a better understanding of the character evolution. In both programs transversional and selected first and second position changes in codon base pairs (i.e., nonsynonymous substitutions) were given extra weight through the use of weighted characters and stepmatrices (see character weighting).

Two separate cladistic analyses were performed using the entire informative data set in which the weighted characters were given the extra weight of 4 and then 8, respectively. Extra weight was given to 20 of the 59 phylogenetically informative characters. These weighted characters included all informative transversions and selected transitional changes (see Fig. 2 and results for explanation) that resulted in amino acid replacement. A single and identical most parsimonious tree (Fig. 6) requiring 187 mutational steps was found in each analysis using the combination of the heuristic searching method of PAUP and MacClade described above.

A second analysis was performed on the subset of the 20 informative weighted substitutions using the branch-bound searching method of PAUP. Twenty-six equally parsimonious trees were found that required 37 mutational steps or less (1 tree required 36 steps). A majority rule consensus cladogram on the right in Figure 6 summarizes these results. A bootstrap analysis of this data set was performed using the same tree searching method with 400 replicates.

RESULTS

CYTOCHROME *b* SEQUENCE EVOLUTION

At least 273 homologous base pairs of a region of the mitochondrial cytochrome *b* (cyt-*b*) gene were sequenced for each of the 32 samples listed on Table 1. Within these 32 samples, 14 species or subspecies were examined and 20 haplotypes found (see Table 2). Ninety-six base pair changes were found at 78 sites in the 19 haplotypes identified for the family Vireonidae. Eleven, 4 and 81 of these changes occurred in first, second and third base pairs of codons respectively. Of the 96 total base pair substitutions, 72 were transitions, 41 of which are shared between taxa and thus phylogenetically informative; 24 were transversions, 18 of which are informative. No other mutational events such as insertions or deletions were observed. In every sample the cyt-*b* gene coded for a complete protein segment of the gene with no stop codon encountered.

CHARACTER ANALYSIS

Transition vs. transversion analysis. In this data set there are three types of characters; silent transitions, silent transversions and first and second position changes in the codon that lead to amino acid changes in the protein. Aquadro and Greenberg (1983), Brown (1985), Edwards and Wilson (1990) have estimated that the ratio of silent transitions to silent transversions ranges from 10:1 to 20:1. This range is supported by the data presented here. Between closely-related species, e.g., *V. olivaceus/V. altiloquus* (11 transitional changes), *V. bellii/V. griseus* (14 transitions and 1 transversion) and *V. gilvus* ssp./*V. philadelphicus* (20 transitions and 1 transversion), a single transversion is accompanied by at least 14 transitions (Table 3). This trend is illustrated in Figure 1 where the number of transitions is plotted against the number of transversions. It appears that transversions are accumulating at a much slower rate and therefore should be more informative phylogenetically than transitions.

Table 2. Cytochrome *b* haplotype sequence data.

	1 1 C	L	I	1 1 T	Q	I	2 1 I	T	G	L	3 1 L	L	A	4 1 M	H	Y	5 1 T	A	D
Pica pica	TGC	CTA	ATT	ACA	CAA	ATC	ATC	ACA	GGC	TTA	CTA	CTA	GCC	ATA	CAT	TAC	ACA	GCA	GAC
Vireolan.l.	...	...	...	...	...	...	G..	...	...	C..	T..	...	...	..G	..C	...	...	...	...
Cyclar.guj.	...	...	...	...	...	...	...	...	..A	C..	...	...	...	.C.	..C	...	...	..C	...
V.g.gilvus A	...	...	G.C	...	...	..T	G..	...	...	C..	...	...	..A	...	..C	...	..T	...	...
V.g.gilvus B	...	...	G.C	...	...	..T	G..	...	...	C..	...	...	..A	...	..C	...	..T	...	...
V.g.gilvus C	...	...	..C	...	...	..T	G..	...	...	C..	...	...	..A	...	..C	...	..T	...	...
V.g.swain.	...	...	...	...	...	..T	G..	...	...	C..	...	...	..A	...	..C	...	..T	...	...
V.philadel.	...	...	...	...	...	...	G..	...	...	C..	..G	...	..A	...	..C	...	..T	...	...
V.altiloquus	...	...	...	...	...	..T	...	...	...	C..	...	...	..A	...	..C	...	..T	...	..T
V.olivac. A	...	...	G.C	...	...	..T	G.T	...	...	C..	...	...	..A	...	..C	...	..T	...	...
V.olivac. B	...	...	G.C	...	...	..T	G.T	...	...	C..	...	...	..A	...	..C	...	..T	...	...
V.bellii A	...	...	..C	...	...	...	...	...	...	C.C	...	...	...	...	..C	...	..C	...	...
V.bellii B	...	...	..C	...	...	...	...	...	...	C.C	...	...	...	...	..C	...	..C	...	...
V.griseus	...	...	..C	...	...	...	G..	...	...	C.C	...	...	...	...	..C	...	..C	...	...
V.atricap.	...	...	..C	...	...	...	G..	...	...	C.C	..G	...	...	...	..C	...	..C	...	...
V.vicinior	...	...	..C	...	...	...	..T	...	...	C.T	...	...	...	..G	..C	...	..T	...	...
V.s.solit. A	...	...	G.C	...	..G	..T	G..	...	...	C.C	..C	...	...	...	..C	...	..C	...	...
V.s.solit. B	...	...	G.C	...	..G	..T	G..	...	...	C.C	..C	...	...	...	..C	...	..C	...	...
V.s.cass.	...	...	..C	...	...	...	G..	...	...	C.C	..T	...	...	...	..C	...	..C	...	...

	6 1 T	S	L	A	7 1 F	A	S	8 1 V	S	H	9 1 M	C	R	N	1 0 1 V	Q	F	1 1 1 G	W
Pica pica	ACC	TCC	CTA	GCC	TTT	GCC	TCA	GTA	TCC	CAC	ATA	TGC	CGT	AAT	GTA	CAA	TTT	GGA	TGA
Vireolan.l.	..T	...	...	...	..C	AA.	..C	...	...	...	...	...	..A	..C	...	...	...	..G	...
Cyclar.guj.	...	...	...	..A	..C	AAT	...	...	G..	...	.C.	...	..A	...	...	..G	..C	...	...
V.g.gilvus A	...	...	...	..A	..C	T..	..T	...	G..	..T	.CT	...	..A	..C	...	...	..C	..C	...
V.g.gilvus B	...	...	...	..A	..C	T..	..T	...	G..	..T	.CT	...	..A	..C	...	...	..C	..C	...
V.g.gilvus C	...	...	...	..A	..C	T..	..T	...	G..	..T	.CT	...	..A	..C	...	...	..C	..C	...
V.g.swain.	...	...	...	..A	..C	T..	..C	...	G..	...	.CC	...	..A	..C	...	...	..C	..C	...
V.philadel.	...	...	...	..A	..C	T..	..C	...	G..	...	.CT	...	..A	...	..G	...	..C	...	...
V.altiloquus	...	...	...	..A	..C	A..	..C	...	G.T	...	..C	...	..A	..C	...	...	...	..	...
V.olivac. A	...	...	...	..A	..C	A..	..T	...	G..	...	.CC	...	..A	..C	..G	...	...	...	...
V.olivac. B	...	...	...	..A	..C	A..	..T	...	G..	...	.CC	...	..A	..C	..G	...	..C	...	...
V.bellii A	..T	...	...	..A	...	...	..T	...	G..	..T	.C.	...	..A	..C	...	...	..C	...	...
V.bellii B	..T	...	...	..A	...	...	..T	...	G..	..T	.C.	...	..A	..C	...	...	..C	...	...
V.griseus	...	...	...	..A	..C	...	..C	...	G..	...	.C.	...	..A	..C	...	...	..C	...	...
V.atricap.	..T	.T.	...	...	..C	...	..C	...	G..	..T	.C.	...	..A	..C	...	...	..C	...	...
V.vicinior	...	...	...	..A	..C	A..	..C	..G	G..	...	.C.	...	..A	..C	...	...	..C	...	...
V.s.solit. A	...	...	...	..A	...	AA.	...	...	G.T	...	...	...	..A	..C	...	..G	..C	...	...
V.s.solit. B	...	...	...	..A	...	AA.	...	...	G.T	...	...	...	..A	..C	...	..G	..C	...	...
V.s.cass.	...	...	...	..A	..C	AA.	...	...	G..	..T	...	...	..A	..C	...	..G	..C	...	...

	1 2 1 L	I	1 2 1 R	N	1 3 1 L	H	A	1 4 1 N	G	A	1 5 1 S	F	F	F	1 6 1 I	C	I	Y	1 7 1 L
Pica pica	CTA	ATC	CGA	AAT	CTC	CAT	GCA	AAC	GGA	GCC	TCC	TTC	TTC	TTC	ATC	TGC	ATT	TAT	CTA
Vireolan.l.	...	...	...	..C	..A	..C	...	...	...	...	..T	..T	...	...	...	...	...	..C	...
Cyclar.guj.	...	...	...	..C	A.T	..C	...	...	..G	...	..A	...	...	...	...	...	..C	..C	...
V.g.gilvus A	...	...	...	..C	..A	..C	...	...	...	...	...	..T	...	...	...	...	..C	..C	...
V.g.gilvus B	...	...	...	..C	..A	..C	...	...	...	...	...	..T	...	...	...	...	..C	..C	...
V.g.gilvus C	...	...	...	..C	..A	..C	...	...	...	...	...	..T	...	...	...	...	..C	..C	...
V.g.swain.	...	...	...	..C	..A	..C	...	...	...	...	...	...	...	...	...	...	..C	..C	..G
V.philadel.	...	..T	...	...	..A	..C	...	...	...	..T	...	...	...	...	...	...	..C	..C	...
V.altiloquus	...	...	...	..C	..A	..C	...	...	...	...	...	...	...	..T	...	...	...	..C	..T

Table 2. Continued.

V.olivac. A	...	...	...	..C	..A	..C	...	...	...	...	...	...	...	..T	...	...	..C	..C	..T
V.olivac. B	...	...	...	..C	..A	..C	...	...	...	...	...	...	...	..T	...	...	..C	..C	..T
V.bellii A	...	...	...	..C	...	...	...	...	...	..T	...	...	...	...	...	...	..C	..C	...
V.bellii B	...	...	...	..C	...	...	...	...	...	..T	...	...	...	...	...	...	..C	..C	...
V.griseus	...	...	...	..C	..T	...	...	...	..G	...	..T	...	...	...	...	...	..C	...	...
V.atricap.	...	...	...	...	..A	..C	...	..T	...	...	...	...	...	..T	...	...	..C	..C	...
V.vicinior	..G	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...	..C	..C	...
V.s.solit. A	...	...	...	...	..A	..C	...	...	...	...	...	...	...	...	...	...	..C	..C	...
V.s.solit. B	...	...	...	...	..A	..C	...	...	...	...	..T	...	...	...	...	...	..C	..C	...
V.s.cass.	...	...	...	...	..A	..C	...	...	...	...	..T	...	...	...	...	...	..C	..C	...

				181			191			201				211			221		
	H	I	G	R	G	F	Y	Y	G	S	Y	L	N	K	E	T	W	N	I
Pica pica	CAC	ATC	GGC	CGA	GGA	TTC	TAC	TAT	GGA	TCA	TAC	CTA	AAT	AAA	GAA	ACC	TGA	AAC	ATC
Vireolan.l.	..T	...	...	...	...	C.A	...	..C	..C	..G	...	...	..C	...	...	...	...	...	..T
Cyclar.guj.	...	...	...	...	...	C.A	...	..C	..C	...	..T	...	..C	...	...	...	...	...	..T
V.g.gilvus A	...	...	...	...	...	C.A	...	..C	...	...	...	...	..C	...	...	..A	...	...	G.A
V.g.gilvus B	...	...	...	...	...	C.A	...	..C	...	...	...	...	..C	...	...	..A	...	...	G.A
V.g.gilvus C	...	...	...	...	...	C.A	...	..C	...	...	...	...	..C	...	...	..A	...	...	G.A
V.g.swain.	...	...	...	...	...	C.A	...	..C	...	...	...	...	..C	...	...	..A	...	...	G.A
V.philadel.	...	...	...	...	...	C.G	...	..C	...	...	...	T..	..C	...	...	..G	...	...	G.A
V.altiloquus	..T	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	...	...	...	...	G.A
V.olivac. A	...	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	...	...	...	...	G.A
V.olivac. B	...	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	...	...	...	...	G.A
V.bellii A	..T	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	...	...	...	...	G.A
V.bellii B	..T	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	...	...	...	...	G.A
V.griseus	...	...	...	...	...	C.A	...	..C	..C	...	...	..G	..C	...	...	...	...	...	G.A
V.atricap.	...	...	...	...	...	C.A	...	..C	..C	...	...	...	...	...	...	..T	...	...	G.A
V.vicinior	...	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	..G	...	...	...	G.A
V.s.solit. A	..T	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	..G	...	...	...	G.A
V.s.solit. B	..T	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	..G	...	...	...	G.A
V.s.cass.	..T	...	...	...	...	C.A	...	..C	..C	...	...	...	..C	...	...	...	...	...	G.A

	231				241			251			261				271
	G	V	I	L	L	L	T	L	M	A	T	A	F	V	G
Pica pica	GGA	GTA	ATC	CTC	CTC	CTA	ACT	CTA	ATA	GCA	ACT	GCT	TTC	GTA	GGA
Vireolan.l.	...	...	...	...	..A	T.C	..C	..T	...	...	...	...	...	...	...
Cyclar.guj.	...	..C	...	..A	..A	...	..C	...	...	...	...	..C	...	...	...
V.g.gilvus A	...	..C	...	..A	..A	...	...	...	...	...	..C	...	...	...	...
V.g.gilvus B	...	A.C	...	..A	..A	...	...	...	...	...	..C	...	...	...	...
V.g.gilvus C	...	..C	...	..A	..A	...	...	...	...	...	..C	...	...	...	...
V.g.swain.	..G	..C	...	..A	..A	...	...	...	...	...	..C	...	...	...	...
V.philadel.	...	..T	..T	..A	..A	...	..C	..G	...	...	..C	...	...	...	..G
V.altiloquus	...	..C	...	...	..A	...	...	...	...	...	...	...	...	...	...
V.olivac. A	...	..C	...	...	..A	...	...	...	...	...	...	...	...	...	..G
V.olivac. B	...	..C	...	...	..A	...	...	...	...	...	...	...	...	...	...
V.bellii A	...	..C	...	...	..A	...	G.C	...	...	...	...	..C	...	..C	...
V.bellii B	...	..C	...	...	..A	...	..C	...	...	...	...	..C	..T	..C	...
V.griseus	...	..C	...	...	..A	...	..C	...	...	..G	...	..C	...	...	...
V.atricap.	...	...	..T	...	..A	...	..C	..G	...	...	..?	...	.?T	..T	...
V.vicinior	..G	...	..T	...	..A	...	..C	..G	...	...	..C	..C	...	...	...
V.s.solit. A	..G	...	...	..A	..A	...	..C	...	..G	...	..C	..C	...	...	..G
V.s.solit. B	..G	...	...	..A	..A	...	..C	..G	..G	...	..C	..C	...	...	..G
V.s.cass.	..G	...	...	..A	..A	...	..C	...	...	...	...	..C	..T	...	...

Identical base pairs are indentified by '.', a change is represented by a letter (A = adenine, C = cytosine, G = guanine and T = thymine) and ambiguities by '?'. The Genbank accession numbers for the above sequences are U12291–U12308.

Table 3. Matrix of pairwise estimates of Kimura's distance (sequence divergence between haplotypes) based on Kimura's '2-parameter' model (1980) (above diagonal) and total number of base pair substitutions followed by number of transversions (below diagonal) for species of Vireonidae and *Pica pica*.

Haplo	Vgg A	Vgg B	Vgg C	Vgs	Vph	Val	Vol A	Vol B	Vbe A	Vbe B	Vgr	Vat	Vvi	Vss A	Vss B	Vsc	C.gu	Vl.le	P.pi
Vgg A	—	.0037	.0037	.0298	.0805	.0815	.0575	.0496	.0947	.0947	.0944	.1075	.1072	.1075	.1159	.0991	.1171	.1345	.1545
Vgg B	1/0	—	.0074	.0336	.0845	.0855	.0614	.0535	.0988	.0988	.0986	.1117	.1114	.1117	.1202	.1033	.1215	.1389	.1591
Vgg C	1/0	2/0	—	.0260	.0765	.0774	.0614	.0535	.0905	.0905	.0903	.1033	.1030	.1117	.1202	.0949	.1215	.1301	.1500
Vgs	8/0	9/0	7/0	—	.0725	.0614	.0614	.0535	.1072	.1072	.0903	.1117	.0947	.1075	.1159	.0991	.1258	.1301	.1455
Vph	21/1	22/1	20/1	19/1	—	.1142	.0976	.0976	.1281	.1281	.1193	.1030	.1111	.1284	.1284	.1199	.1473	.1562	.1541
Val	21/6	22/6	20/6	16/6	29/5	—	.0450	.0450	.0978	.0978	.0976	.1232	.1061	.1111	.1196	.1027	.1295	.1033	.1270
Vol A	15/5	16/5	16/5	16/5	25/5	11/0	—	.0074	.0978	.0978	.0894	.1148	.0978	.1069	.1153	.1069	.1165	.1288	.1447
Vol B	13/5	14/5	14/5	14/5	25/5	11/0	2/0	—	.0896	.0896	.0813	.1064	.0896	.1069	.1153	.0986	.1080	.1288	.1492
Vbe A	24/10	25/10	23/10	27/10	32/9	25/6	24/6	24/6	—	.0074	.0568	.0889	.0807	.1022	.1106	.0776	.1117	.1288	.1307
Vbe B	24/10	25/10	25/10	27/10	33/9	25/6	24/6	24/6	2/0	—	.0568	.0809	.0807	.1022	.1106	.0696	.1117	.1288	.1307
Vgr	24/9	25/9	23/9	23/9	30/8	25/5	23/5	21/5	15/1	15/1	—	.0932	.0765	.1061	.1061	.0734	.0905	.1199	.1304
Vat	27/11	28/11	26/11	29/11	26/10	30/7	27/7	25/7	22/3	20/3	34/4	—	.0971	.1187	.1187	.0855	.1562	.1321	.1524
Vvi	26/10	27/10	25/10	24/10	29/9	27/6	24/6	22/6	21/2	21/2	20/2	25/3	—	.1017	.1017	.0935	.1202	.1281	.1476
Vss A	27/11	28/11	28/11	27/11	32/10	29/9	28/9	28/9	26/7	26/7	27/5	30/5	26/5	—	.0074	.0489	.1156	.1414	.1623
Vss B	29/11	30/11	30/11	29/11	32/10	31/9	30/9	30/9	28/7	28/7	27/5	30/5	26/5	2/0	—	.0489	.1199	.1371	.1714
Vsc	25/11	26/11	24/11	25/11	31/10	27/9	27/9	25/9	20/6	18/6	19/6	21/6	23/5	13/0	13/0	—	.0947	.1072	.1488
C.gu	29/15	30/15	30/15	31/15	36/14	32/13	29/13	27/13	27/11	27/11	23/10	37/14	30/11	31/11	32/11	24/10	—	.1335	.1488
Vl.le	33/14	34/14	32/14	32/16	38/14	28/11	31/12	29/12	31/9	31/9	30/10	32/8	33/9	35/10	34/10	30/10	33/12	—	.1253
P.pi	36/16	37/16	37/16	35/14	38/15	31/14	36/14	36/14	31/12	31/12	32/10	36/11	36/10	40/13	42/13	36/12	37/13	32/9	—

See Table 1 for haplotype acronyms.

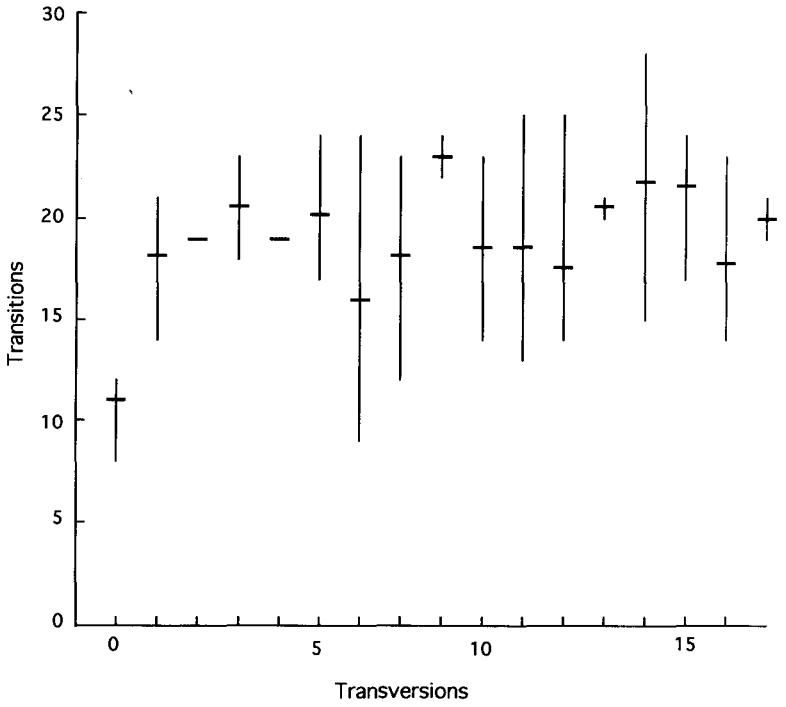

FIGURE 1. Observed transitional vs. transversional nucleotide substitutions. Data were gathered from every pairwise comparison. The solid horizontal bar represents the mean. The vertical bar represents the range.

Studies of the mitochondrial rRNA of artiodactyls and mammalian cytochrome *b* have shown that transversions are accumulating in a linear manner over time scales much greater than those considered here (Miyamoto and Boyle 1989, Irwin et al. 1991). Although informative at the early stages of divergence, the rapid rate of transition accumulation quickly clouds their linear trail with parallel and reversion events once a plateau of divergence is reached (Irwin et al. 1991). As a result of these differing rates of accumulation, many investigators have used transversions and substitutions causing amino acid changes, exclusively, in the construction of their trees for taxa separated at relatively high taxonomic levels (Meyer and Wilson 1990, Irwin et al. 1991).

Amino acid analysis. In these taxa, a total of 16, first and second positions within a codon display base pair changes which lead to 12 amino acid changes (Fig. 2). Six of these amino acid changes are phylogenetically informative. The conservative change of valine to isoleucine occurs in three of these informative amino acid changes (see Fig. 2). Both valine and isoleucine contain nonpolar aliphatic side chains and are relatively unreactive. Furthermore in the molecule studied, these changes all occur in transmembrane regions (Howell 1989), are all the result of transitions, and in the case of the substitution at position 7 (Table 2), are highly variable. At this position, variation is observed within one subspecies (*V. g. gilvus*) and also between closely related taxa. For example, position 7 varies between the subspecies pair *V. g. gilvus* and *V. g. swainsonii* and also between the subspecies pair *V. s. solitarius* and *V. s. cassinii*. For these reasons, this amino acid change did not receive additional weight in the cladistic analyses. The remaining three informative positions, caused by five nucleotide substitutions, were either transversions or transitions that lead to a change from a polar to nonpolar amino acid. These changes were weighted as described above in the methods for cladistic analyses.

ESTIMATES OF PHYLOGENY

Phenetic analysis. The Neighbor-Joining distance tree (Fig. 3), derived from Kimura's distances (Table 3), contains a monophyletic *Vireo*

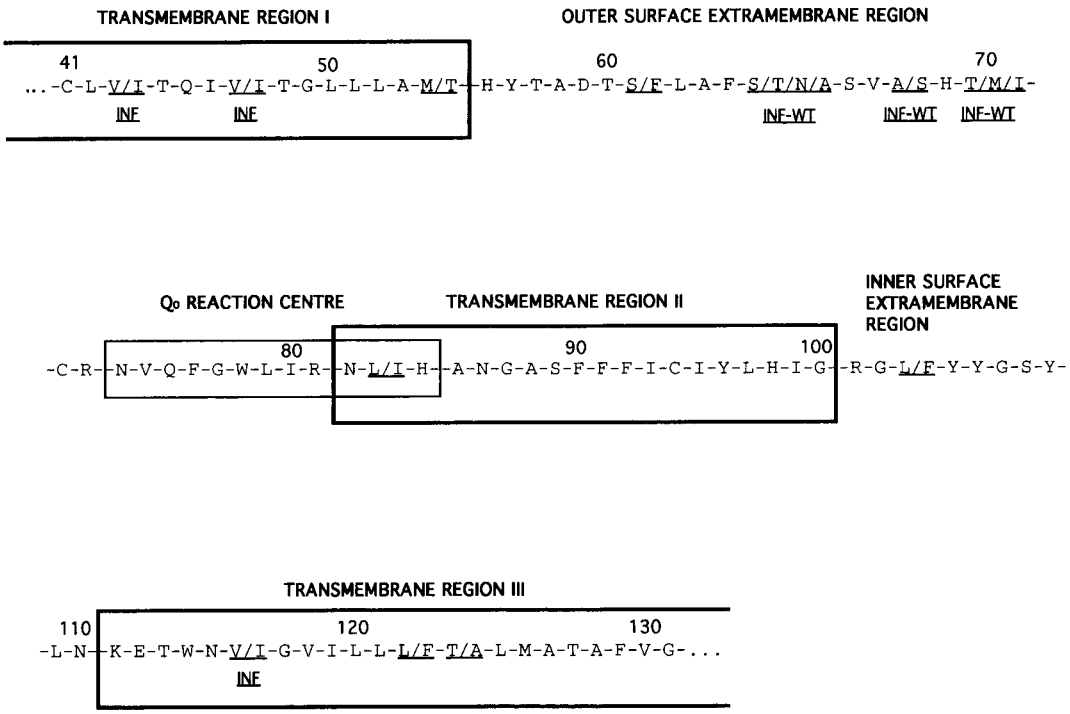

FIGURE 2. The amino acid sequence variation of the region of cytochrome *b* analyzed in relation to putative transmembrane and Q_0 reactions (Howell 1989). Twelve variable amino acid sites are identified by underlining. At most, four and usually two amino acids were variable at these sites. Sites with *INF* underneath are phylogenetically informative. Sites with *INF-WT* underneath are the weighted informative sites used to construct the cladograms in Figure 6. The numbers correspond to homologous sequence of chicken mitochondrial cytochrome *b* (Desjardins and Morais 1990).

clade. Within the *Vireo* clade the *V. solitarius* ssp. are ancestral to all other vireos which radiate in two primary lineages. The first lineage contains all eye-lined vireos sampled (subgenus *Vireosylva*) while the second contains most of the eye-ringed vireos (subgenera *Vireo* and *Lanivireo*). As mentioned previously, the bootstrap values are accepted as confidence levels for nodes in the phylogeny. In this tree, bootstrap values are considerably lower for the deeper branch points. Only the relationships between individuals of the same species and between subspecies can be viewed with confidence.

The Fitch-Margoliash distance tree (Fig. 4) is similar to the Neighbor-Joining tree (Fig. 3). A clear distinction remains between the eye-lined and eye-ringed vireos. Two major differences involve the placement of the *V. solitarius* ssp. and *C. gujanensis* within the eye-ringed clade. Additionally, the placement of *V. atricapillus* and *V. vicinior* is reversed. In both trees, the confidence of these branches is low.

Maximum likelihood analysis. The Maximum Likelihood phylogeny (Fig. 5) again contains separate clades of eye-lined and eye-ringed vireos. Like the Fitch-Margoliash estimate (Fig. 4) this tree also contains *V. solitarius* ssp. and *C. gujanensis* within the eye-ringed clade. This clade, however, differs from the other phylogenies in the position of *V. atricapillus.*

Cladistic analyses. The results of this analysis found in the weighted cladogram (Fig. 6, left-hand tree) are thought to represent the best approximation of the phylogeny of this clade because of the combined use of weighted and transitional characters. The entire data set was used because the transitions still contain some information about the more recent branch points. In this way it was hoped the ambiguities found within the eye-ringed group would be removed.

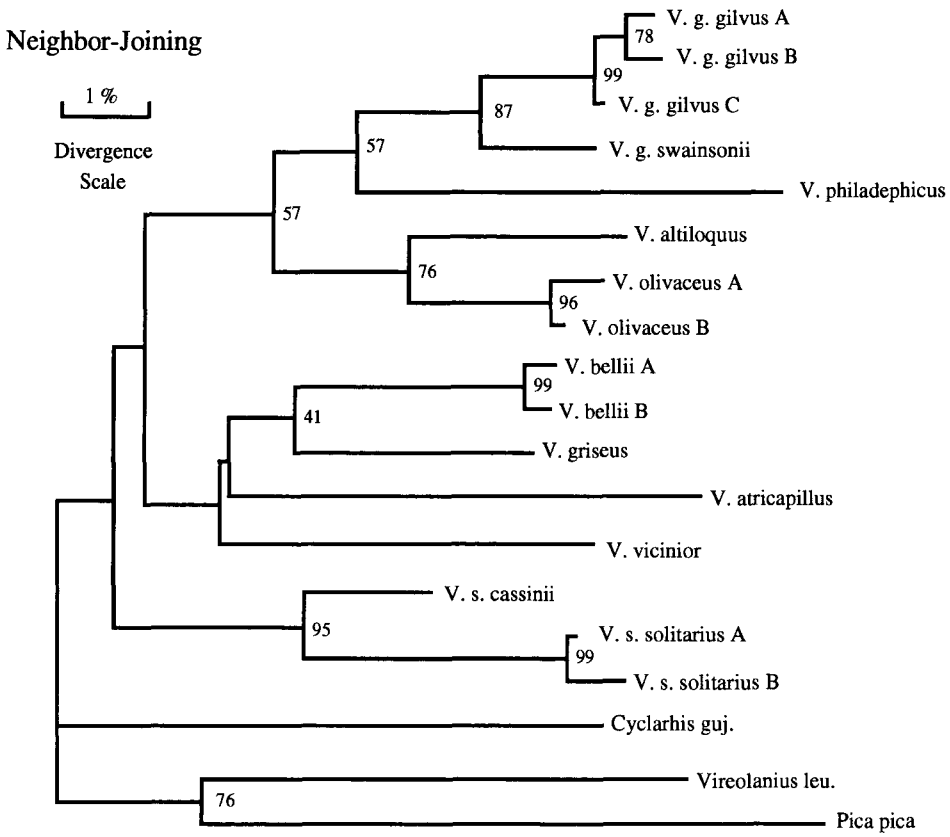

FIGURE 3. Neighbor-Joining distance tree. The tree was constructed from the pairwise distance estimates in Table 3 utilizing the computer program Neighbor contained in the phylogenetic package Phylip 3.5c (Felsenstein 1993). Percent divergence is represented by the total horizontal distance between terminal tips and can be estimated using the scale. The number next to each node is the number of bootstrap trees out of 1,000 that contain this branching pattern.

A single most parsimonious tree was found in two separate analyses. The first analysis was conducted with the proportional weight of 4 for the weighted characters (see Character Analysis). The second analysis was conducted with the proportional weight of 8. This tree shows a monophyletic *Vireo* clade radiating in two distinct lineages (Fig. 5). Again there is a clear distinction between the eye-lined and eye-ringed vireos. The branching arrangement of the eye-lined vireos is identical in all trees (Figs. 3, 4, 5, 6). In all these cases the eye-lined vireos are subdivided into two groups. One group contains *V. gilvus* ssp. and *V. philadelphicus* while the other contains *V. olivaceus* and *V. altiloquus*. Within the eye-ringed group a major point of ambiguity is the placement of *V. vicinior* and *V. atricapillus*. In this cladogram these two species are found in the same clade as the *V. solitarius* ssp. The species *V. griseus* and *V. bellii* group consistently in pairs (Figs. 3, 4, 5, 6).

A majority rule consensus cladogram summarizes the results of an analysis using the weighted characters only (Fig. 6, right-hand tree). This analysis was conducted to reveal the phylogenetic information contained within the subset of 20 weighted characters. Although only the branching pattern within the eye-lined vireo clade is supported by the bootstrap analysis, the consistency index of the weighted characters explaining the most parsimonious trees (0.6) is higher than that resulting from a transition analysis (0.421). The 26 cladograms which this consensus cladogram represents all have a consis-

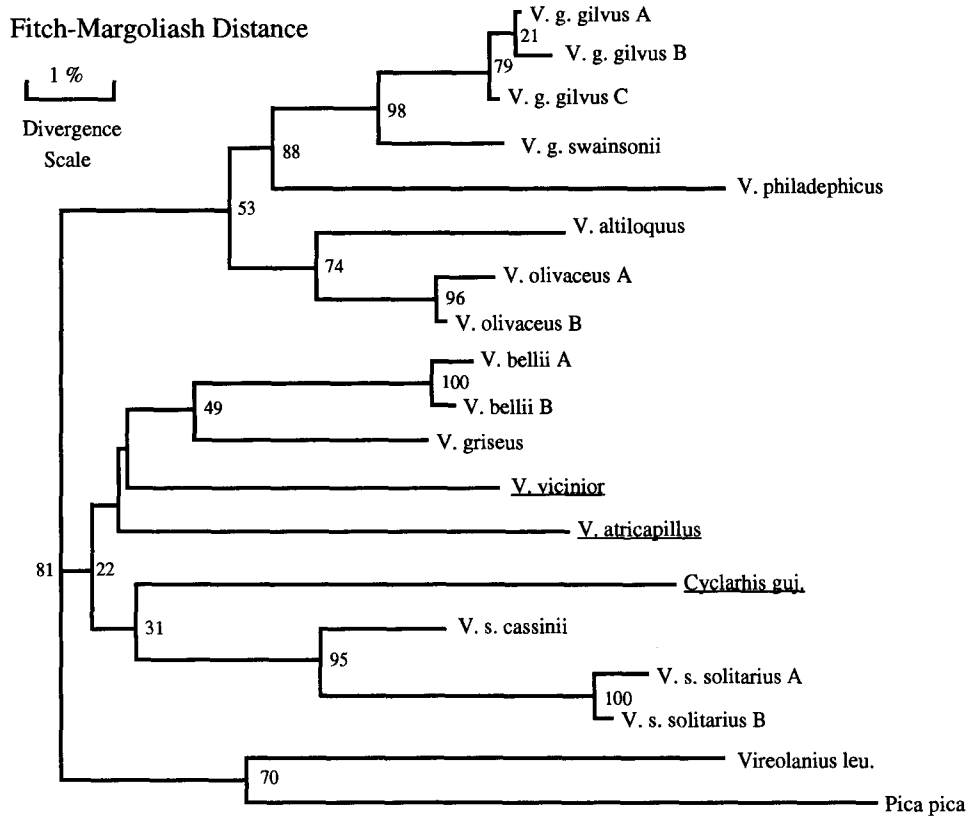

FIGURE 4. Fitch-Margoliash distance tree constructed from the pairwise distance estimates in Table 3 utilizing the computer program Fitch contained in the phylogenetic package Phylip 3.5c (Felsenstein 1993). Percent divergence is represented by the total horizontal distance between terminal tips and can be estimated using the scale. Numbers located at the nodes indicate the number of bootstrap trees out of 1,000 that contain this branching pattern. The underlined taxa differ in their placement within the tree from the Neighbor-Joining analysis.

tency index (C.I.) of approximately 0.6 (ranges from 0 to 1; 1 indicates all characters are consistent with the given tree). In a separate analysis only transition character changes, a subset of 38 characters, were used to estimate phylogeny (results not shown). In this case, the C.I. of the most parsimonious trees was 0.421. From this result it can be argued that the weighted characters (transversions and selected nonsynonymous substitutions) contain a more consistent record of the phylogeny of the species under examination than the transitional events.

DISCUSSION

GENERIC RELATIONSHIPS

In all analyses, *V. leucotis* is found to be the most diverged member of the Vireonidae examined. This is consistent with monophyly of the genus *Vireo*. The position of *C. gujanensis* is less clear. Although the exact position of the Rufous-browed Peppershrike is unclear, it is more closely related to *Vireo* than the Slaty-capped Shrike-vireo. In two analyses, the Fitch-Margoliash distance tree and the Maximum Likelihood tree, the genus *Vireo* appears to be polyphyletic. In these two trees, *C. gujanensis* is situated next to the subspecies of *V. solitarius*. Alternatively, monophyly in *Vireo* is supported by the weighted cladograms and the Neighbor-Joining distance tree. Bootstrap analysis and the variability of results indicate an uncertainty in the placement of *C. gujanensis* within the phylogeny of the present species. Further sequence analysis and the addition of more vireo species or subspecies (e.g., *V. solitarius plumbeus*) are needed to resolve the question of monophyly within the genus *Vireo*.

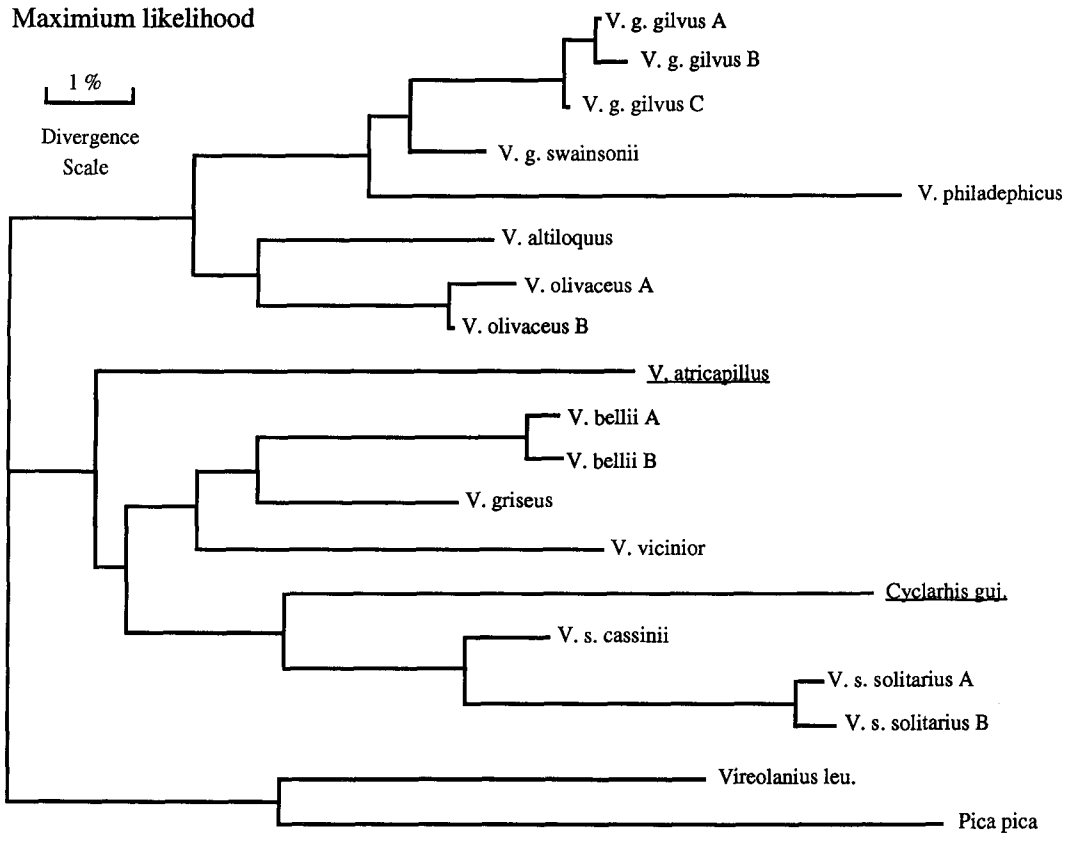

FIGURE 5. Maximum likelihood estimate of the phylogeny. This tree was estimated utilizing the DNAml program contained within the phylogenetic package Phylip 3.5c (Felsenstein 1993). Percent divergence is represented by the total horizontal distance between terminal tips and can be estimated using the scale. The ln of this tree is −1319.36. The underlined taxa differ in their placement within the tree from the Neighbor-Joining analysis.

SPECIES RELATIONSHIPS WITH *VIREO*

All trees show a clear division of the two major clades in the genus *Vireo*: the eye-lined forms (*Vireosylva*) and the eye-ringed forms (*Vireo* and *Lanivireo*). This result is best illustrated by the number of transversions between and within the groups. The greatest number of transversions between species within a given clade is 6 while the number of transversions between the clades ranges from 5 to 11, averaging 8.2. In all analyses, supposed members of the subgenus *Lanivireo* are as closely related to species of the subgenus *Vireo* as they are to each other indicating that *Lanivireo* is not a valid taxon as suggested by Johnson et al. (1988).

Within eye-lined vireos, the branching order is identical in all estimates of phylogeny (Figs. 3, 4, 5, 6). The subdivision of this clade into *V. gilvus/philadelphicus* and *V. olivaceus/altiloquus* groups was also noted by Johnson et al. (1988). Previous work on the Philadelphia Vireo agrees with the phylogenetic relationship described here. Johnson et al. (1988), Barlow (unpubl. allozyme data) and Hamilton (1962) found *V. gilvus* and *V. philadelphicus* to be closely aligned in their analyses. The close phylogenetic relationship of the phenotypically similar Red-eyed and Black-whiskered vireos is not unexpected. DNA-DNA hybridization studies also show these two species to be very closely related (Sibley and Ahlquist 1982).

The phylogenies suggest that a proto eye-lined vireo gave rise to two forms; proto red-eyed and

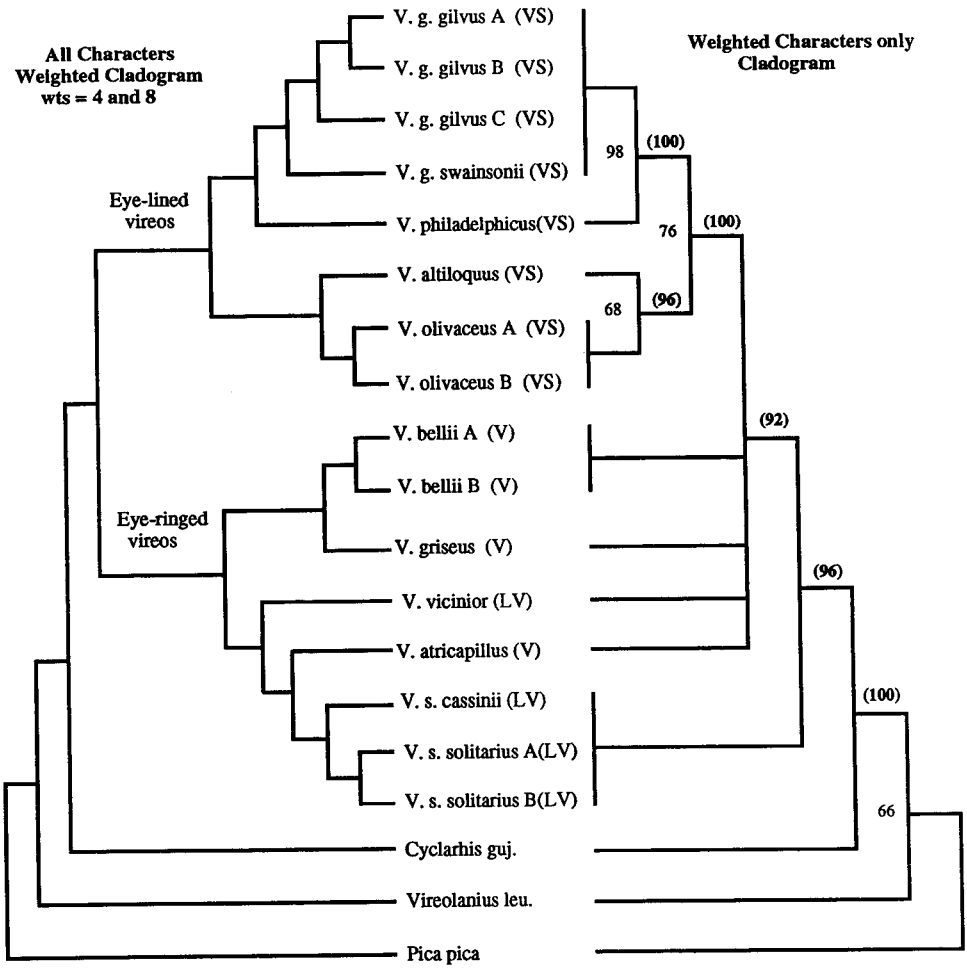

FIGURE 6. The left-hand cladogram is the single most parsimonious tree (i.e., shortest total length) when phylogenetically informative transversional and selected nonsynonymous substitutions were given weights of four and eight respectively. It requires 187 mutational steps. Both trees were found utilizing the branch-bound search procedure of the phylogenetic computer program PAUP 3.0 (g) (Swofford 1989). The majority rule consensus cladogram to the right of the species names was derived using only the weighted characters. Twenty-six equally parsimonious trees requiring 37 mutational steps or less (one tree was found to require 36 steps) were found. The bold numbers along the branches indicate the percentage of trees that possessed the given branch point. The bracketed numbers at the nodes indicate the percentage of bootstrap trees (out of 400) that contained the given branch point. Nodes without numbers indicate that the branch point was not found in at least 50% of the bootstrap trees.

gilvus. Such a proto eye-lined form may be reflected in the Golden Vireo (*V. hypochryseus*) of central Mexico which possesses the requisite eye-line and a quavering monosyllabic song (Barlow 1990), but in which nest building is shared by both sexes (Barlow, unpubl. data). *V. gilvus* males occasionally assist in the earliest stages of nest construction (Howes-Jones 1985), whereas male *V. olivaceus* and *V. philadelphicus* do not assist at any stage. The proto red-eyed vireo speciated further into *V. olivaceus* and *V. altiloquus*. Meanwhile, the proto *gilvus* form had given rise to the *V. gilvus* ssp. and *V. philadelphicus*. This agrees with Barlow's (1980) hypothesized species radiation in which a postulated proto *Vireosylva* gave rise in Central America to the proto *gilvus* and proto red-eyed forms which subsequently invaded North America to establish breeding

populations. This interpretation is disputed by Johnson and Zink (1984) based on allozyme data in a study centered on the Yellow-green Vireo (*V. flavoriridis*), which suggests that a *V. olivaceus* type arose in South America.

The eye-ringed vireos make up the second major clade. All phylogenies except the Neighbor-Joining tree (Fig. 3) support this clade. The remaining trees (Figs. 4, 5, 6) suggest an ancestral proto eye-ringed vireo has given rise to all subsequent forms. There appear to be two major groups within this clade: the *V. belli, V. griseus* group and the *V. solitarius* group. The *V. solitarius* clade is supported strongly in the bootstrap analyses (95% Fig. 3; 95% Fig. 4). The *V. bellii, V. griseus* clade is not as strongly supported by the bootstrap analyses (41% Fig. 3; 49% Fig. 4), but is found consistently in all trees.

The relationships of the Black-capped (*V. atricapillus*) and Gray (*V. vicinior*) vireos within this complex has the weakest bootstrap support. Further assay may be warranted before the relationships of *V. vicinior* and *V. atricapillus* to other vireos of the subgenus *Vireo* can be clarified. In most analyses, however, *V. vicinior* appears more closely aligned with the *bellii-griseus* group while *V. atricapillus* is closer to the *solitarius* group with the position of the root within this clade remaining unclear. In either case the intermediate positions of *V. vicinior* and *V. atricapillus* in this clade are also reflected in the behavioral data. For example, the song and certain courtship displays of *V. vicinior* resemble those of *V. solitarius plumbeus* (Barlow et al. 1970), whereas in foraging behavior and choice of scrub habitat *V. vicinior* is similar to *V. griseus* and *V. bellii. V. atricapillus* also closely resembles *V. griseus* and *V. bellii* in foraging behavior and choice of low shrubby habitat in the breeding season (Barlow 1990), but share the feature of a distinctive 'cap' in both sexes with northern *V. solitarius* ssp. of this study.

One of the clearer relationships within the eye-ringed clade is the association of the Bell's and White-eyed vireos. This result is consistent with phylogenetic interpretations discussed by Hamilton (1962) and Orenstein and Barlow (1981), but is at a variance with phylogenetic interpretations for these taxa by Johnson et al. (1988). With a sample size of two *V. bellii* these authors showed that *V. bellii* was highly diverged from any other *Vireo* species. However, Barlow (unpubl. allozyme data) found that *V. bellii* ($n = 7$) and *V. griseus* ($n = 5$) showed a level of relatedness paralleling results for these taxa in our mtDNA study.

This electrophoretic result of Johnson et al. (1988), which is in conflict with our DNA sequence results, may be due to the differences in the assumptions of the two techniques. In assessing protein variation, the homology of the characters (electromorphs) is not known to the same level of certainty as DNA sequence variation. Furthermore, the degree of genetic difference between the electromorphs is unknown. Evolutionary events such as genetic bottlenecks may lead to fixed allele differences which result in an overestimate of genetic difference. For example the large number of fixed allele differences of *V. bellii* (Johnson et al. 1988) but low sequence differences (this study) may be the result of genetic bottleneck or similar phenomena.

METHODOLOGY

The low bootstrap confidences of the branches in the eye-ringed clade may indicate a number of methodological problems. First, the length of sequence examined is only 273 bp. Martin et al. (1990) investigated variation in genetic estimates in relation to the size of homologous sequences compared. They reported that variation in estimates of genetic distance increases as the area of homologous DNA examined decreases. This may explain the difference in confidence levels between the eye-lined and eye-ringed clades. The eye-lined clade contains two groups, the *olivaceus* and *gilvus* groups, which appear to have evolved relatively early in the history of the clade and then, only fairly recently, speciated into the taxa examined here. The first split, characterized by at least five transversional events, is followed by the more recent speciation of *V. gilvus* and *V. philadelphicus,* characterized by one transversional event, and *V. olivaceus* and *V. altiloquus,* having no transversional events (see Table 3). These types of widely spaced and thus highly characterized evolutionary events are likely to be observable in analyses of small regions of DNA. Conversely, the eye-ringed clade seems to have undergone a more uniform speciation process. This is illustrated by a pairwise comparison of transversional events between the member taxa which ranges evenly between one and seven. Because the evolution of this clade has been more uniform, including a number of relatively contemporaneous speciation events, it is possible

that speciation events are blurred by the two processes of random parallel nucleotide substitution or reversions and, thus, the pitfall of examining a small region of homologous DNA. Additional sequencing of other regions of the mtDNA would be required to test this hypothesis and improve the estimation of the phylogeny of the eye-ringed clade.

Small sample sizes for each species may have led to the low confidence values for the eye-ringed clade. The seemingly contradictory characters that lead to low confidence values for the bootstrap analyses may be simply the retention of ancestral polymorphisms. Because the sample sizes used here are small, this possibility cannot be addressed. It seems unlikely, however, as geographic variation in mtDNA is low in other North American birds (Ball et al. 1988, Moore et al. 1991).

A final problem with this study is that only nine of the 26 currently recognized species of *Vireo* were included in this survey. The addition of the remaining species of *Vireo* would help to clarify some species relationships. One strength of comparing sequence data is that samples once obtained and sequenced can be added easily to the data base for reanalysis. This is clearly a future direction for investigation of vireo systematics.

UTILITY OF CYTOCHROME *b* FOR PHYLOGENTIC INFERENCE

The amount of variation present in the portion of cyt-*b* sequence analyzed has proven sufficient for an estimation of *Vireo* phylogeny. This type of survey is suitable for comparisons ranging from generic (Irwin et al. 1991) to subspecific (Edwards and Wilson 1990; this study). We recommend analysis of a longer region of cyt-*b* than conducted here for the analysis of species of separate genera. Further, the low amount of variation observed within subspecies, which may be indicative of the entire mitochondrial genome in birds, would not recommend a cyt-*b* comparison for populations below the rank of subspecies. However, a study by Johnson and Cicero (1991) found cyt-*b* analysis useful for assessing relationships between populations of three Sage Sparrow (*Amphispiza belli*) subspecies. In this case an average percent nucleotide difference of 0.6% was observed between the subspecies. The population survey revealed the geographical intermediate *A. b. canescens* subspecies to contain a cyt-*b* sequence, in one population, identical to that found in *A. b. belli,* and a second cyt-*b* sequence, in another population, identical to that found in *A. b. nevadensis.*

For some comparisons at the subspecific level, cyt-*b* results are useful in identifying different mitochondrial haploypes and in estimating genetic distances between them. However, cyt-*b* is only nominally informative about population interactions between the subspecies. Cyt-*b* may reveal possible haplotype introgression (indicative of hybridization) but field observations or nuclear DNA data are needed to supplement this finding. This is a drawback not only of cyt-*b* data but of all mtDNA analyses, because only a single maternal marker is present in the offspring.

The rate of nucleotide substitution in cyt-*b* shows a bias of at least 10 transitions to one transversion. Although not necessarily a problem when comparing closely related species, this fact must be considered when interpreting results at higher taxonomic levels. The greater the relative evolutionary time between comparison of species the higher the likelihood of parallel or reversed nucleotide substitutions which in turn may obscure phylogenetic relationships. This problem is obviously greatest when analyzing the rapidly accumulating transitions. Thus, the slower accumulating transversions and substitutions causing amino acid changes should contain a more accurate record of phylogeny than more rapidly accumulating transitions, and should be weighted accordingly.

CONCLUSIONS

Cytochrome *b* sequence analysis has proven a useful tool for reconstructing the phylogenetic history of *Vireo. Vireo* is made up of two major lineages: the eye-lined vireos (subgenus *Vireosylva*) and the eye-ringed vireos (subgenera *Vireo* and *Lanivireo*). In contrast to Johnson et al. (1988), we still recommend the use of part of the subgeneric classification. We do not, however, find support for the subdivision of eye-ringed vireos into the subgenera *Vireo* and *Lanivireo.* Further work is warranted to establish the validity of the taxon *Lanivireo.*

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