SPECIATION IN SAPSUCKERS (Sphyrapicus):
III. MITOCHONDRIAL-DNA SEQUENCE DIVERGENCE AT THE
CYTOCHROME-B LOCUS

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ABSTRACT.—We amplified and sequenced a 711 base-pair (bp) fragment of mtDNA at the
cytochrome-b locus to reexamine relationships within and among species of Sphyrapicus and
representatives of two other woodpecker genera (Melanerpes, Colaptes). Sequences were ob-
tained from 10 individuals of 5 taxa: Red-breasted Sapsucker (S. ruber daggetti), n = 2; Red-
naped Sapsucker (S. nuchalis), n = 2; hybrid of S. ruber daggetti × S. nuchalis, n = 1; Yellow-
bellied Sapsucker (S. varius varius), n = 2; Williamson's Sapsucker (S. thyroideus thyroideus), n = 2; and Red-bellied Woodpecker (M. carolinus), n = 1. The Melanerpes sequence and a
comparable published sequence of Andean Flicker (C. rupicola) were used as outgroups. Levels
of mtDNA sequence divergence ranged from 0.0 to 0.6% within species and 0.1 to 10.2%
among species of Sphyrapicus, and from 12.5 to 14.5% between members of Sphyrapicus and
the two outgroup species. One region of sequence (78 bp) was identified as being especially
conserved. Branching topologies based on this study corroborated results from a previous
analysis of relationships in Sphyrapicus using allozymes: the phenotypically dissimilar species
ruber and nuchalis are most closely related; varius, which is very similar in appearance to
nuchalis, branches off next; and thyroideus, the most divergent member of the quartet from
the standpoint of plumage pattern and degree of sexual dimorphism, is strongly differentiated
genetically. Pairwise mtDNA distances within Sphyrapicus showed a strong curvilinear re-
lationship with allozyme distances (r = 0.996). The monophyly of Sphyrapicus is reaffirmed,
with S. thyroideus closer to the ancestral species. Members of Sphyrapicus are more similar to
Melanerpes than to Colaptes based on their mtDNA. Prior hypotheses regarding the evolu-
tionary history of sapsuckers in North America are discussed. Despite the near genetic identity
of ruber and nuchalis based on both allozymes and mtDNA sequences, and their tendency for
limited hybridization in sympatry, an earlier study of mating preference supports the biologic-
species status of these taxa. Received 1 November 1994, accepted 1 March 1995.

ALTHOUGH UNDOUBTED CONGENERS, the four
phenotypically distinct forms of Sphyrapicus have
drawn the attention of avian systematists re-
peatedly over the past half century (Short 1982).
Grinnell and Miller (1944:236), for example,
treated the Red-breasted Sapsucker (S. ruber) and
Red-naped Sapsucker (S. nuchalis) as subspecies
of S. varius, despite their pronounced plumage
differences and narrowly sympatric distribu-
tions, because the two former taxa were "pec-
cularly mixed and apparently to some extent
interbreeding" in the Warner Mountains, Mor-
oc County, California. Howell (1952), working
in the same region, discovered the first nests of
mixed pairs. He also expanded the known range
of sympatry between ruber and nuchalis to in-
Clad Mono County, California, where interbreeding is “apparently extremely slight” (Howell 1952:251). In addition, Howell examined variation in the third taxon, the Yellow-bellied Sapsucker (S. varius), which closely resembles nuchalis in appearance, but nests allopatrically in northern and eastern North America. Recognizing the similar natural histories and essential allopatry of all three taxa, and the limited hybridization of at least ruber and nuchalis, Howell (1952) combined them into one species, S. varius.

Johnson and Zink (1983) published the first genetic (i.e. allozyme) information for Sphyrapicus, which revealed that the phenotypically similar forms nuchalis and varius are not sister taxa. Surprisingly, nuchalis was nearly identical genetically to ruber, while varius and S. thyroideus (Williamson’s Sapsucker) were distinctive allozymically, the latter taxon strongly so. These results further suggested that ruber and nuchalis should be considered conspecific, but that varius is a full species. The species status of thyroideus has never been questioned because of its unique phenotype and extreme sexual dimorphism relative to other members of the genus. Short and Morony (1970) and Johnson and Zink (1983) commented on the phylogeny of Sphyrapicus based on phenotypes and allozymes, respectively.

Most recently, Johnson and Johnson (1985) closely examined the relationship of ruber and nuchalis through a detailed study of microdistribution and mating preference of sympatric nesting pairs in southern Oregon, eastern California, and western Nevada. Despite a relatively low level of hybridization, these authors found that the majority of matings were positively assortative. The preponderance of conspecific matings in sympathy, combined with apparent selection against F hybrids (Johnson and Johnson 1985), provided crucial evidence for the biologic species status of ruber and nuchalis despite their near genetic identity as revealed by protein electrophoresis (Johnson and Zink 1983).

In the present study, we reexamined the puzzling relationships of sapsuckers in the genus Sphyrapicus using direct sequencing of mtDNA. This technique not only has the potential to clarify the taxonomy of this group, but it also can provide the basis for strong phylogenetic inference (Avise 1994, Hillis et al. 1994). For several reasons, DNA sequence data from conspecific species can contribute fundamental information to an understanding of the processes of molecular evolution. First, nucleotide differences among undoubted close relatives provide a valuable baseline against which sequences of increasingly divergent taxa can be assessed. Second, the gradual accumulation of sequence data for avian congeners—e.g. Pomatostomus (Edwards and Wilson 1990, Edwards et al. 1991), Amphispiza (Johnson and Cicero 1991), Laniarius (Smith et al. 1991), Uria (Birt-Friesen et al. 1992), Phylloscopus (Richman and Price 1992), and Sphyrapicus (present study)—permits identification of conserved and variable regions at early stages of genetic change, still an exploratory topic. Third, DNA sequence information for taxa that also have been studied allozymically provides a useful comparative perspective. Finally, we are the first to provide sequence data for an interspecific hybrid.

**Materials and Methods**

Specimens examined.—Sequences were obtained from two specimens of each of the four species of Sphyrapicus currently recognized by the AOU (1983, 1985): Red-breasted Sapsucker (S. ruber daggetti) from Fresno County, California; Red-naped Sapsucker (S. nuchalis), Okanogan County, Washington; Yellow-bellied Sapsucker (S. varius varius), Franklin County, New York (n = 1) and Ontario, Canada (n = 1); and Williamson’s Sapsucker (S. thyroideus thyroideus), Wasco County, Oregon (n = 1) and Okanogan County, Washington (n = 1). We also sequenced a putative F hybrid between S. ruber daggetti and S. nuchalis from the Warner Mountains, Lake County, Oregon, the same individual examined by Johnson and Zink (1983) in their electrophoretic study. We chose a species of Melanerpes (Red-bellied Woodpecker [M. carolinus], Latimer County, Oklahoma) as one outgroup because of Short and Morony’s (1970) hypothesis that the ancestor of Sphyrapicus stemmed from a melanerpine woodpecker line. A sequence of another outgroup species, the Andean Flicker (Colaptes rupicola), was obtained from the literature (Edwards et al. 1991).

**DNA techniques.**—A 10- to 20-mg sample of frozen liver tissue was taken with a sterile blade from each taxon, washed three times in 1 ml cold 1× STE buffer (0.1 M NaCl; 10 mM Tris, pH 8.0; 1 mM EDTA), and digested overnight in 500 µl of lysis buffer (50 mM Tris HCL, pH 8.0; 50 mM ethylenediamine tetracetate [EDTA], pH 8.0; 1% sodium dodecyl sulfate; 100 mM NaCl; 1% 2-mercaptoethanol) and 11 µl proteinase K. Tubes were incubated at 55°C and gently rocked until all of the tissue was dissolved. RNase A (5.5 µl) was added to each sample 1 h before the end of incubation. Whole-genomic DNA was extracted once with phenol (pH 8.0), once with phenol:SEVAG (1:
Double-stranded (dsPCR) and single-stranded (ssPCR) amplifications were performed using different combinations of light (L) and heavy (H) strand primer pairs (Table 1) to obtain approximately 711-bp sequences for each taxon. Each dsPCR reaction (25 μl total volume) contained 12.5 μl of the target DNA (diluted by 1:100 in water) and 12.5 μl of a mixture of 1× TAQ buffer (Cetus Corp.: 10 mM Tris [pH 8.3], 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin; or modified New England Biolab [NEB] 0.067 M Tris [pH 8.8], 2 mM MgCl₂, 0.0167 M ammonium sulfate, 0.01 M beta-mercapto ethanol), 0.75 mM dNTP mix, 1 μM of each primer, 0.625 units of Thermus aquaticus (Taq) polymerase, and double-distilled water. Each cycle of amplification involved denaturation at 92° to 93°C for 1 min, annealing at 50°C for 1.5 min (30-32 cycles); the first cycle was preceded by an initial denaturation step for 3 min at 92° to 93°C, and the last cycle was followed by a final extension for 3 min at 72°C. Plugs of amplified double-stranded DNA were excised from agarose minigels (3% HGT/1% Nusieve, 1× TAE buffer) and by staining with ethidium bromide (10 μg/ml). Double-stranded products were cleaned of free nucleotides and excess salts by spinning the samples for 4 min (1,650 rpm) using Quick-Spin G-50 Sephadex columns (Boehringer Mannhelm Corp.) with pre-swollen beads. Prior to loading the DNA, columns were spun twice for 1 and 2 min, respectively. Sequencing reactions were performed using 7 μl of DNA template and the primer that was limiting in ssPCR amplifications. A commercial kit (Sequenase, US Biochemical Corp.) was used for sequencing according to the Sanger dideoxy chain-termination method (Sanger et al. 1977). Products from the sequencing reactions were loaded onto 6% polyacrylamide-8.3 M urea linear gels (1× TBE buffer), run for 1.5 to 5 h at 40 to 45°C, and autoradiographed.

Data analysis.—DNA sequences were entered into a Macintosh computer using MacDNASYS Pro, version 1.0 (Hitachi Software Engineering America, Ltd. 1991), then aligned and translated according to the mammalian genetic code. The sequence of Colaptes rupicola is a corrected version of that presented by Edwards et al. (1991), which differs in its translation by one amino acid (human: codon number 83, nucleotide number 14995; chicken: codon number 84, nucleotide number 15144). While the published se-

<table>
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<th>Primer location</th>
<th>Primer sequence</th>
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<tr>
<td>L14987</td>
<td>5'-CCATCCACATCTG(A/T)GG(A/T)GATG-3'</td>
</tr>
<tr>
<td>L15236</td>
<td>5'-TACCTAACAAGAAACG(T/C)TGATG-3'</td>
</tr>
<tr>
<td>L15321</td>
<td>5'-TGGAGGAAATAATG(A/C)TCGAGG-3'</td>
</tr>
<tr>
<td>H15304</td>
<td>5'-GTAGCACCCTG(A/G/T)AATTTG-3'</td>
</tr>
<tr>
<td>H15557</td>
<td>5'-GACTGTGACAAAATCCC(G/A/T/C)TTCCA-3'</td>
</tr>
<tr>
<td>H15570</td>
<td>5'-ATTGCGATAGG(A/G/T/C)AT(T/C)ATGATG-3'</td>
</tr>
</tbody>
</table>

* Letters refer to light (L) and heavy (H) strands of mtDNA; numbers correspond to location of 3' end of primer in chicken (Gallus) sequence (Desjardins and Morais 1990).

* Degenerate sites indicated by brackets.
Fig. 1. Sequences of a 711-bp fragment of the cytochrome-\(b\) gene for four species of Sphyrapicus and two outgroups, Melanerpes carolinus and Colaptes rupicola. Dots indicate sequence identity to C. rupicola. One-letter abbreviations correspond to Colaptes amino-acid sequence. Codon number (above sequences) and nucleotide number (below sequences) refer to cytochrome-\(b\) sequence in chicken (Desjardins and Morais 1990). N = not determined.
C. rupicola
N. carolinus
S. ruber 1
Hybrid
S. michaels 1
S. michaels 2
S. varius 1
S. varius 2
S. thyroides 1
S. thyroides 2
C. rupicola
N. carolinus
S. ruber 1
Hybrid
S. michaels 1
S. michaels 2
S. varius 1
S. varius 2
S. thyroides 1
S. thyroides 2

Fig. 1. Continued.

sequence has a codon of CAA (=glutamine), three other C. rupicola sequences have a codon of CAC (=histidine) at that position (W. S. Moore in litt.). Furthermore, all other avian cytochrome-b sequences that we have examined (e.g. Desjardins and Morais 1990, Johnson and Cicero 1991, Edwards et al. 1991 [all non-Colaptes sequences], Richman and Price 1992, Kra- jewski and Fetzner 1994, Lanyon and Hall 1994). As well as amphibian (e.g. Moritz et al. 1992, Graybeal 1993) and mammal (e.g. Anderson et al. 1981, Bibb et al. 1981, Howell 1989, Smith and Patton 1991) sequences, also share histidine at that homologous codon. Reliability of the two varius sequences was corroborated by comparison with an overlapping, essentially identical sequence of the same taxon from Chicago, Illinois (Lanyon and Hall 1994).

MacLink Plus (version 6.0) converted the sequence data from a Macintosh to a DOS file for analysis on an IBM personal computer (PC) or equivalent. Basic sequence statistics (percent nucleotide composition by codon position, transitions versus transversions, silent versus replacement substitutions, percent sequence difference) and pairwise distance estimates (Kimura [1980] two-parameter distance, Tamura-Nei [1993] distance) were computed using the program MEGA (Molecular Evolutionary Genetics Analysis, version 1.01; Kumar et al. 1993). Underdetermined sites were ignored in pairwise comparisons. Although Kimura's distance method is the most widely cited measure for nucleotide data, we prefer the Tamura-Nei model for avian sequences because it does not assume equal nucleotide frequencies (0.25) throughout the evolutionary process (for detailed discussion of methods of distance estimation for sequence data, see Kumar et al. 1993:15-30). Variable and conserved regions of sequence were identified using MEGA by nonoverlapping window analysis of 3-bp (=1-codon) segments. The relative frequency of changes between different nucleotide states (G, A, T, C) was determined using MacClade (Maddison and Maddison 1992).

To test for saturation effects, we regressed the proportion of transitional and transversional substitutions at each codon position against Tamura-Nei distances between pairs of sequences. Based on this analysis, transitions and transversions at all codon positions were included without weighting in the phylogenetic analysis. Prior to tree construction, a frequency distribution of 1,000 randomly sampled trees was obtained using PAUP (Phylogenetic Analysis Using Parsimony, version 3.1.1; Swofford 1993) to assess the strength of "phylogenetic signal" versus random noise among the sequences (see Hillis 1991, Huelsenbeck 1991, Hillis and Huelsenbeck 1992). Relationships within Sphyrapicus and between Sphyrapicus and each of the two outgroups were examined by maximum-parsimony and neighbor-joining analyses with 1,000 bootstrap replications (PAUP and MEGA programs, respectively). The branch-and-bound method of parsimony analysis was used, and both
strict and 50% majority-rule consensus trees were obtained. The neighbor-joining analysis was based on Tamura-Nei distances. Concordance between patterns of mtDNA variation (this study) and allozyme variation (Johnson and Zink 1983) within Sphyrapicus was tested statistically by pairwise correlation analysis of genetic distances (Tamura-Nei’s D versus Nei’s D, respectively).

RESULTS

PATTERNS OF mtDNA VARIATION AND EVOLUTION

Levels of sequence divergence.—Sequences for the eight samples of Sphyrapicus, one hybrid S. ruber daggetti × S. nuchalis, and two outgroups are provided in Figure 1. Translation and alignment identified 711 homologous nucleotides shared by two or more individuals. Of these sites, 161 (22.6%) were variable between at least two samples representing the genera examined (Sphyrapicus, Melanerpes, Colaptes) and 81 were variable within Sphyrapicus. Levels of sequence divergence ranged from 0.0-0.6% within species to 0.1-10.2% among species of Sphyrapicus, and from 12.5-14.5% between members of Sphyrapicus and the two outgroup genera (Table 2). Sphyrapicus ruber and S. nuchalis were least divergent (0.1-0.7%), while S. varius varied from the ruber-nuchalis complex by 2.0-2.3%. The hybrid sequence was identical to that of the two ruber samples. Sphyrapicus thyroideus was strongly divergent from other sapsuckers (9.8-10.2%) but, of the four species of Sphyrapicus, differed least (12.5%) from Melanerpes. Within Sphyrapicus, the only intraspecific differences occurred between the two specimens of nuchalis (0.6%), both of which were collected at the same locality. Individuals of varius and thyroideus from different localities showed no geographic variation. Larger sample sizes are needed to adequately assess levels and patterns of intraspecific variation in the cytochrome-b sequences of these taxa.

Tamura-Nei (1993) distances were essentially identical to values of percent sequence difference in comparisons of ruber, nuchalis, varius, and the hybrid (Table 2). Although the Tamura-Nei method gave slightly larger values for thyroideus and the two outgroups, the general trend remained consistent. The more popular method of estimating pairwise nucleotide distances using Kimura’s (1980) two-parameter model gave similar results.

Table 3 compares percent sequence divergence between species of Sphyrapicus with levels found in other avian genera for which cytochrome-b sequences have been published. The number of nucleotides analyzed in our study is clearly comparable to those in other investigations. Average divergence among species within a genus varied from 3.3% between certain cranes (e.g. in Anthropoides) to 12.9% in barbets (Capito). With the exception of Sphyrapicus, species in genera of lower taxonomic orders (e.g. Gruiformes, Charadriiformes) generally showed less divergence than near-passerine (Piciformes) or passerine taxa. The relatively low value for mean sequence divergence in Sphyrapicus (5.8%) is attributed to the near genetic identity of ruber and nuchalis (average difference = 0.4%, the lowest value reported for any pairwise comparison of congeneric species). Divergence between these taxa and varius (2.0-2.3%; Table 2) also was low compared to
TABLE 3. Comparisons of mtDNA sequence divergence (cytochrome b) within 10 genera representing four orders and eight families or subfamilies of birds. Data for genera other than Sphyrapicus (this study) calculated from published sequences¹.

<table>
<thead>
<tr>
<th>Genus</th>
<th>No. species compared</th>
<th>No. pairwise comparisons</th>
<th>No. nucleotides</th>
<th>$\bar{x} \pm SE^b$ (range$^b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gruiformes (Gruidae)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Balearica</td>
<td>2</td>
<td>1</td>
<td>1,042</td>
<td>$0.041 \pm 0.006$ (--)</td>
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<tr>
<td>Anthropoides</td>
<td>2</td>
<td>1</td>
<td>1,042</td>
<td>$0.033 \pm 0.006$ (--)</td>
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<td>Grus</td>
<td>10</td>
<td>84</td>
<td>1,042</td>
<td>$0.055 \pm 0.007$ (0.013-0.077)</td>
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<td>Charadriiformes (Alcidae)</td>
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<tr>
<td>Uria</td>
<td>2</td>
<td>17</td>
<td>307</td>
<td>$0.066 \pm 0.014$ (0.059-0.072)</td>
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<td>Piciformes (Capitonidae)</td>
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<tr>
<td>Capito</td>
<td>2</td>
<td>1</td>
<td>888</td>
<td>$0.129 \pm 0.011$ (--)</td>
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<td>Piciformes (Picidae)</td>
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<tr>
<td>Sphyrapicus</td>
<td>4</td>
<td>9</td>
<td>711</td>
<td>$0.058 \pm 0.008$ (0.004-0.102)</td>
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<td>Laniarius</td>
<td>6</td>
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<td>295</td>
<td>$0.092 \pm 0.017$ (0.058-0.124)</td>
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<td>Passeriformes (Timaliinae)</td>
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<td>Pomatostomus</td>
<td>5</td>
<td>176</td>
<td>282</td>
<td>$0.097 \pm 0.018$ (0.050-0.128)</td>
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<td>Phylloscopus</td>
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<td>169</td>
<td>910</td>
<td>$0.114 \pm 0.011$ (0.049-0.142)</td>
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<td>Passeriformes (Emberizinae)</td>
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<tr>
<td>Amphispiza</td>
<td>2</td>
<td>14</td>
<td>288</td>
<td>$0.105 \pm 0.018$ (0.101-0.109)</td>
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</tbody>
</table>


² Values given for all pairwise comparisons between species within a genus, including geographic samples of populations and/or subspecies where analyzed.

other species, although it fell within the range found among species of Grus. In contrast, the average divergence (9.9%) between thyroideus and the ruber-nuchalis-varius complex is similar to that found between other congeneric species of Piciformes or Passeriformes.

Nucleotide composition of sequences.—Figure 2 illustrates the percent frequency of nucleotides at different codon positions in cytochrome-b sequences of Sphyrapicus, Melanerpes, and Colaptes. While first position sites are comprised of approximately equal proportions of the four nucleotides, strong compositional biases are noted at second and third positions of codons. Second positions are clearly biased toward thymine (T) and against guanine (G). Most striking is the bias toward cytosine and deficiency of guanine at third position (i.e. silent) sites. While adenine, thymine, and cytosine showed a wide range of values at third positions, especially compared to first and second positions, the low proportion of guanine at silent sites was more consistent among all avian sequences examined. Sequences of Sphyrapicus, Melanerpes, and
Table 4. Matrix of substitutions between pairs of cytochrome-\(b\) sequences (711 bp) for four taxa of Sphyrapicus (\(n = 2\)), one hybrid S. ruber daggetti \(\times\) S. nuchalis, and two outgroups (Melanerpes carolinus [\(n = 1\]), Colaptes rupicola*, [\(n = 1\)]. Ratios of transitions: transversions given above the diagonal; ratios of silent: replacement changes given below the diagonal. Substitutions between two haplotypes of S. nuchalis are indicated.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>5</th>
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<td>1 S. ruber</td>
<td>--</td>
<td>0:0</td>
<td>0:1</td>
<td>4:1</td>
<td>15:1</td>
<td>58:11</td>
<td>63:31</td>
<td>63:36</td>
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<tr>
<td>2 Hybrid</td>
<td>0:0</td>
<td>--</td>
<td>0:1</td>
<td>4:1</td>
<td>15:1</td>
<td>58:11</td>
<td>63:31</td>
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</tr>
<tr>
<td>3 S. nuchalis 1</td>
<td>1:0</td>
<td>1:0</td>
<td>--</td>
<td>4:0</td>
<td>14:0</td>
<td>57:13</td>
<td>62:30</td>
<td>61:36</td>
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<tr>
<td>4 S. nuchalis 2</td>
<td>5:0</td>
<td>5:0</td>
<td>4:0</td>
<td>--</td>
<td>15:0</td>
<td>59:13</td>
<td>63:30</td>
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<td>5 S. varius</td>
<td>16:0</td>
<td>16:0</td>
<td>14:0</td>
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<td>7 M. carolinus</td>
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<td>86:7</td>
<td>79:9</td>
<td>--</td>
<td>65:35</td>
</tr>
<tr>
<td>8 C. rupicola</td>
<td>89:10</td>
<td>89:9</td>
<td>88:9</td>
<td>89:9</td>
<td>80:9</td>
<td>90:12</td>
<td>87:13</td>
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Colaptes had a relatively high proportion of thymine at silent sites compared to other avian taxa.

Patterns of substitution among sequences.—The majority of interspecific substitutions within Sphyrapicus were transitions at silent sites (Table 4). Of the transversions and replacement substitutions observed at the intrageneric level, most occurred between thyroideus and the other three congeners (including the hybrid). The total number of substitutions, as well as the proportion of substitutions comprised of transversions and/or replacement changes, increased progressively from the ruber-nuchalis-varius complex to thyroideus, Melanerpes, and Colaptes.

Transitions between pairs of sequences were dominated by thymine-cytosine or cytosine-thymine substitutions (Fig. 3). In contrast, changes between guanine and either thymine or cytosine were relatively rare in our samples, especially when outgroups were excluded from the analysis. No guanine-cytosine or cytosine-guanine transversions were observed between species within Sphyrapicus.

Conserved regions of sequences.—Cytochrome-\(b\) sequences of Sphyrapicus, Melanerpes, and Colaptes revealed one region of 26 codons (78 bp) that was highly conserved within Sphyrapicus (Fig. 4), with only one substitution occurring in that segment (see also Fig. 1). Although comparisons between Sphyrapicus and either Melanerpes and/or Colaptes did not show a similar pattern, this is an artifact attributed to our inclusion of all substitutions. If we limit the analysis to replacement substitutions, the protein sequences of Melanerpes and Colaptes are similarly conserved in that region (only one amino-acid difference between Colaptes and all other sequences). A smaller segment of 33 bp (codons 199-209) also was identified as being conserved within Sphyrapicus. The most stable regions among all sequences occurred between codons 199-203 (15 bp), 217-219 (9 bp), and 253-258 (18 bp), where no substitutions were noted (Figs. 1 and 4).

Analysis of Phylogenetic Relationships

Lack of saturation effects.—The proportion of pairwise substitutions comprised of transitions and transversions at first and second positions showed a slow but steady increase with mtDNA distance (Fig. 5). Third-position transitions also increased linearly, although the rate of change was much faster. These data indicate that our sequences are not plagued by saturation or “multiple-hit” effects at third-position sites. Thus, we had no reason either to exclude or to weigh differentially transitions in our analysis of phylogenetic relationships.

In contrast to transitions, third-position transversions showed a different, curvilinear pattern, whereby the proportion of transversions increased rapidly at a distance of approximately 0.07 to 0.08 (Fig. 5). This threshold is slightly below the average distance of 0.11 between thyroideus and other species of Sphyrapicus. The sharp increase reflects an essential lack of transversions among ruber, nuchalis, and varius when compared to thyroideus, Melanerpes, and Colaptes.

Phylogenetic signal versus random noise.—Analyses of phylogenetic relationships should be based on a relatively strong contribution of signal versus noise in the data set. Recent studies
(Hillis 1991, Huelsenbeck 1991, Hillis and Huelsenbeck 1992) have shown that such information can be obtained by using maximum-parsimony techniques to examine the shape of distributional curves for tree-length frequencies (i.e. by plotting number of trees versus tree length, whereby trees are sampled randomly n times from the set of all possible trees). Curves that are highly skewed to the left indicate strong phylogenetic signal because only a few possible solutions exist for the optimal (=shortest) tree. The degree of skewness (and thus phylogenetic signal) can be tested mathematically using $g_1$ statistics (Sokal and Rohlf 1981, Hillis 1991).

We randomly sampled 1,000 trees from our mtDNA data set, which yielded a strongly left-skewed pattern of tree-length distributions (results not shown). Tree lengths ranged from 259 to 367 steps, with a mean of 341 ± SD of 22 steps. The $g_1$ statistic gave a highly significant value of $-1.256$ ($P < 0.01$; see Hillis 1991:288).

**Branching topologies.**—Maximum-parsimony and neighbor-joining analyses yielded identical tree topologies (Fig. 6). The phenotypically distinct ruber and nuchalis emerged as sister taxa, whereas varius, which closely resembles nuchalis in appearance, fell outside of this group. The female hybrid between S. ruber daggetti and S. nuchalis clearly allied with other ruber. Sphyrapicus ruber, S. nuchalis, and S. varius form a superspecies complex that is highly differentiated from the basal member of the genus, S. thyroideus. The relationships among these four taxa is strongly supported by bootstrapping. Sphyrapicus formed a monophyletic clade compared to Melanerpes and Colaptes, with moderate support in the maximum-parsimony tree (61%) and relatively strong support in the neighbor-joining tree (89%). The lack of a high bootstrap value separating Melanerpes from S. thyroideus and its congeners in the parsimony analysis suggest a closer relationship between Melanerpes and Sphyrapicus (especially thyroideus) than between Melanerpes and Colaptes.

**Congruence between allozyme and mtDNA data.**—A qualitative comparison of our trees with those reconstructed from allozyme data (Johnson and Zink 1983:878) revealed identical patterns of relationship among congeneric species of Sphyrapicus. Like the mtDNA sequence data, Johnson and Zink's examination of nuclear genes showed that ruber and nuchalis form a clade separate from varius, and that the ruber-nuchalis-

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**Fig. 3.** Relative frequency of changes between nucleotides indicated by size and position of circles. State changes reconstructed using parsimony analysis (PAUP) and MacClade; circles denoting maximum and minimum values calculated based on entire set of most-parsimonious reconstructions. Separate analyses performed using all sequences (top) and Sphyrapicus sequences only (bottom).

varius complex is strongly divergent from thyroideus. The hybrid individual analyzed in both studies also allied with ruber based on allozymes. Because Johnson and Zink (1983) did
Within *Sphyrapicus*  
*Sphyrapicus* plus outgroups

![Graph showing sequence variability analysis](image)

**Fig. 4.** Moving-window analysis of sequence variability along a 234-codon (702-bp) segment of the cytochrome-b gene for *Sphyrapicus* and outgroups; silent and replacement sites were included in the analysis. Numbers along horizontal axis refer to codon number in chicken sequence (Desjardins and Morais 1990). Analysis based on nonoverlapping segments of one codon (3 bp) each. Graph shows variability in three-codon blocks; terminal three codons excluded because of undetermined sites (see Fig. 1). Black bars denote substitutions within *Sphyrapicus*; open bars indicate substitutions between *Sphyrapicus* and outgroups or between outgroups. Hatched bars below graph indicate the most conserved regions of sequence within *Sphyrapicus* (left hatching) or between *Sphyrapicus* and outgroups (right hatching).

not include outgroups, their analysis shed no light on the relationship of *Sphyrapicus* to other woodpecker genera.

In order to quantitatively assess concordance between the allozyme and mtDNA data sets, we regressed Nei's (1978) genetic distances (Johnson and Zink 1983:877) against Tamura-Nei (1993) distances (present study) for pairwise comparisons of congenic species within *Sphyrapicus* (Fig. 7). Distance estimates derived for different samples of a species were averaged to give one value for that taxon. Nei's D showed a nearly perfect, curvilinear relationship ($r = 0.996$) with mtDNA distance (Fig. 7). The close congruence exhibited by allozymes and mtDNA sequences for *Sphyrapicus* lends strong support to our conclusions concerning phylogenetic relationships of species in this genus.

**Discussion and Conclusions**

*Patterns of mtDNA sequence variation in* Sphyrapicus *corroborate those shown by previous studies of birds.*—In comparing patterns of mtDNA sequence divergence for different taxonomic groups, Johnson and Cicero (1990) noted a declining ratio of transitional to transversional substitutions from the intrapopulation to interordinal level. They did not include intergeneric/intrafamilial comparisons in their analysis, but reported average ratios of 3.6 for interspecific congeners (*Amphispiza bellii* vs. *A. bilineata*) and 1.0 to 1.2 for interfamilial comparisons. Values for *Sphyrapicus* varied from 2.0 when comparing *ruber* and *nuchalis* to 14.8 among these taxa and *variatus*; the average ratio between *thyroideus* and other species of *Sphyrapicus* was 4.8.
These ratios span the entire range of average values found for avian congeners listed in Table 3 (from 2.1 in Phylloscopus to 15.8 in Pomatostomus; unpubl. data). Intergeneric/intrafamilial comparisons among Sphyrapicus, Melanerpes, and Colaptes yielded slightly lower ratios (\(\bar{x} = 1.7-2.0\)), which fall between those reported by Johnson and Cicero (1990) for interspecific congeners and interfamilial genera. The sharp rise in transversional substitutions with increasing mtDNA distance that we observed among these woodpecker genera lends further support to the trend described above.

The pattern of nucleotide sequence composition observed in Sphyrapicus, Melanerpes, and Colaptes at different codon positions also generally matches that reported for other avian taxa (e.g. Edwards et al. 1991, Johnson and Cicero 1991, Lanyon and Hall 1994). While a strong bias against guanine at silent (third-position) sites is characteristic of vertebrate mtDNA, a moderate bias against thymine at third positions appears to distinguish birds from other vertebrates (Kocher et al. 1989). Likewise, we observed a bias against thymine at silent sites, although the proportion of thymine at third positions was high in sequences of Sphyrapicus, Melanerpes, and Colaptes compared to other birds (Edwards et al. 1991, Johnson and Cicero 1991, Lanyon and Hall 1994, Cicero unpubl. data). The wide range of values shown for adenine, thymine, and cytosine at third positions of codons indicates that the base composition of silent sites may vary considerably among species (see also Lanyon and Hall 1994:391-393).

Differences in base composition at first, second, and third positions of codons have important consequences for patterns of nucleotide substitutions at various taxonomic levels (Kocher et al. 1989:6199-6200). For example, because silent sites in the cytochrome-b sequences of birds were noted to have a strikingly low proportion of thymine relative to other vertebrates, Kocher et al. (1989:6199) stated that “a corresponding deficiency exists in the frequency of thymine to cytosine changes during bird evolution.” Contrary to this expectation, we found a relatively high frequency of thymine-cytosine and cytosine-thymine substitutions in our sequences. This discrepancy may be attributed, at least partly, to the higher percentage of thymine at silent sites in our bird sequences compared to those (11.3%) of Kocher et al. (1989).

Conserved regions of cytochrome b are shared among widely divergent taxa. — An analysis of regional variability within genes, especially protein-coding genes such as cytochrome b, may reveal segments that are conserved during evolution, presumably because they have crucial physiological functions. For example, Howell (1989) found five conserved regions of cytochrome-b protein sequences in both prokaryotes and eukaryotes that appear to be important in redox catalysis. Two of these regions were especially highly conserved (codons 130-150 and 270-290 based on yeast cytochrome b). Howell did not include any bird sequences in his analysis (the only vertebrate was a mouse), and there is only one previous study in which
Fig. 6. Maximum-parsimony and neighbor-joining trees showing relationships within Sphyrapicus, and between Sphyrapicus and each of two outgroups (Melanerpes carolinus, Colaptes rupicola). Parsimony 50% majority-rule consensus tree was derived from three equally short trees of 215 steps (branch-and-bound search); consistency index for tree is 0.971. A strict-consensus tree gave the same topology. Numbers above branches indicate bootstrap values for 1,000 replicates. Sapsucker heads on upper panel illustrate male phenotypes; heads on lower panel illustrate female phenotypes. Note the female hybrid specimen.
patterns of variability within cytochrome \( b \) have been examined for birds (Edwards et al. 1991). Importantly, these authors found the same regions to be stable in terms of replacement substitutions, with the latter segment (270-290) being most conservative.

We did not sequence the conservative segment 270-290 discussed by Howell (1989) and Edwards et al. (1991). However, our study likewise revealed a highly conserved protein region of 26 codons (78-bp) in the cytochrome-b sequences of Sphyrapicus, Melanerpes, and Colaptes. An exciting finding is that this segment occurs between codons 125 and 150 (same numbering for both yeast [Howell 1989] and chicken [Desjardins and Morais 1990] protein sequences). The fact that this region was identified as conservative in three independent studies of widely divergent taxonomic groups using different methodologies for assessing sequence variability provides indirect support for an indispensable metabolic function. Differences in substitution rate within as well as between genes have important implications for phylogenetic analysis.

mtDNA evidence supports conclusion that phenotypically similar forms of Sphyrapicus are not sister taxa.—The new mtDNA data offered here firmly corroborate the finding based on allozymes (Johnson and Zink 1983) that the two most phenotypically similar forms of Sphyrapicus are not sister species. Rather, S. nuchalis, which closely resembles S. varius in its striped head pattern and black breast band, is clearly allied genetically to the red-headed and red-breasted form S. ruber. Limited hybridization between ruber and nuchalis in sympathy provides further evidence of their close relationship. Analysis of the cytochrome-\( b \) sequence for one female hybrid revealed that the maternal parent of this individual was a ruber; this in contrast to Johnson and Johnson's (1985:12) finding that, of nine interspecific matings between pure parental ruber and nuchalis, the female was nuchalis in eight instances.

Evolutionary relationships and taxonomy within Sphyrapicus are well established.—Howell (1952) presented the first hypothesis regarding the evolutionary history of sapsuckers in which he proposed, based on anatomical similarities, that the ancestor of Sphyrapicus was a Dendrocopus or Dendrocopus-like woodpecker. Within the S. varius complex, Howell envisioned a group of continuously interbreeding populations across North America during at least the Lower Pliocene that varied clinally in plumage pattern from the eastern varius phenotype to the western ruber type. In the Middle and Upper Pliocene, changes in topography, climate, and vegetation led to disjunction of these populations into three isolated groups: one east of the Great Plains (pre-variuss), one in the Rocky Mountain region (pre-nuchalis), and one in the Sierra-Cascade and Coast Ranges (pre-ruber and daggetti). Subsequent isolation and contact of these differentiating or differentiated forms probably occurred during glacial and interglacial cycles, respectively. Post-Pleistocene dispersal of variuss, nuchalis, and ruber likely accounts for current geographic distributions and secondary contacts. Howell (1952:279) did not postulate which of these forms is closest to the putative ancestral type, arguing that "any of the modern subspecies [in the varius complex] could have been derived from any of the others through minor and cumulative genetic changes." Because Howell did not examine variation in S. thyroideus, he excluded this species from his scenario for the evolutionary history of the genus.
A different hypothesis for the evolution of all forms of North American sapsuckers was proposed by Short and Morony (1970) based on their analysis of a hybrid between *thyroideus* × *nuchalis*. Using evidence from plumage patterns, geographic distribution, and ecology, these authors envisioned two major splitting events: (1) divergence of an ancestral *Sphyrapicus* stock from a melanerpine woodpecker line (among modern species, *S. thyroideus* was thought to most closely resemble the ancestral phenotype); and (2) splitting of a continuous ancestral population into two isolates, one in western North America (pre-*thyroideus*) and one in eastern North America (pre-*varius*-*nuchalis*-*ruber*). Westward dispersal of the *varius* complex subsequently gave rise to *nuchalis* and *ruber* although, according to Short and Morony (1970: 313), this divergence was recent and the three taxa "are barely specifically distinct." In view of two known hybrids between *thyroideus* and *nuchalis* (Oberholser 1930, Short and Morony 1970:313), the latter authors further concluded that "*thyroideus* is very closely related to the *nuchalis* complex and that interactions between them have affected the evolution of their distinctive plumages." Finally, Short and Morony (1970) questioned whether coexistence of as many as three species of sapsuckers is possible given their similar ecological requirements and tendency for hybridization (however rare).

Johnson and Zink (1983), using evidence from protein electrophoresis, refuted Short and Morony's (1970) hypothetical scenario on several grounds: (1) genetic distances among *ruber*, *nuchalis*, and *varius* clearly showed that *ruber* and *nuchalis* are more closely related to each other than either is to *varius*; (2) genetic distances estimated by allozymes were as different between *thyroideus* and its congeners as they are among species in different wood-warbler genera (Barrowclough 1980); and (3) because three taxa (*ruber*, *nuchalis*, and *thyroideus*) coexist locally (e.g. in the Warner Mountains of extreme south-central Oregon and northeastern California), their basically allopatric distributions probably reflect adaptation to different environmental regimes rather than separation resulting from interspecific competition. Johnson and Zink (1983) did not discuss Howell's (1952) hypothesis, nor did they attempt to resolve the conflict between Howell and Short and Morony (1970) regarding the ancestry of *Sphyrapicus* (i.e. *Dendrocopus* vs. *Melanerpes*). However, another electrophoretic study of woodpeckers (Lanyon and Zink 1987) failed to find a consistently supported branching topology for intergeneric relationships among *Sphyrapicus*, *Picoides* (=*Dendrocopus*), *Melanerpes*, and *Colaptes*.

Our mtDNA data firmly establish the conclusions presented by Johnson and Zink (1983) concerning evolutionary relationships among North American sapsuckers. In particular, the nearly perfect association between genetic distances estimated by allozymes and mtDNA sequences provides incontrovertible evidence that *ruber* and *nuchalis* shared a very recent common ancestor, and that these two taxa, along with *varius*, comprise a fairly young superspecies. The placement of *thyroideus* as the oldest taxon within *Sphyrapicus*, and its deep divergence relative to other members of the genus, also is well established by both data sets. Although the mtDNA data revealed a closer relationship between *Sphyrapicus* and *Melanerpes* than *Colaptes*, sequence analysis of additional woodpecker genera (especially *Picoides*) is needed to definitively resolve relationships at this level.

Despite the near genetic identity of *ruber* and *nuchalis* based on both allozymes and mtDNA, and their tendency for limited hybridization in sympatry, we support the conclusion derived from mating behavior (Johnson and Johnson 1985) that these two taxa are biologic species. The dominance of conspecific matings where they coexist, combined with apparent selection against F hybrids, attests to their essential reproductive isolation. Furthermore, the wide range of values for percent sequence divergence exhibited by different congeners (Table 3) indicates that absolute values of divergence are a poor taxonomic yardstick. Probable genetic mechanisms responsible for the color change from a *nuchalis* to a *ruber* phenotype are discussed by Howell (1952) and Johnson and Johnson (1985).

Lineages are estimated to be older based on mtDNA data compared to allozyme data.—Using a calibration based on Nei's (1978) genetic distance (*t* = 26.3 × 10^6*D*; Gutiérrez et al. 1983), Johnson and Zink (1983) estimated the average date of divergence (*t*) between *thyroideus* and other species of *Sphyrapicus* as approximately 3.7 to 3.0 MYBP. This estimate is erroneous, however, because *nuchalis* was inadvertently omitted from the calculations. A corrected value using the same calibration (*D* = 0.142-0.197) is 5.2 to 3.7 MYBP (i.e. early to mid-Pliocene). Johnson and
Zink (1983) did not estimate divergence time for the ruber-nuchalis split because of the low genetic distances between these taxa, nor did they date the splitting event between varius and the other two taxa in this superspecies complex. Based on an average Nei’s (1978) D of 0.019 to 0.029 between varius and ruber-nuchalis, however, and using the same calibration as Johnson and Zink (1983), we estimate this split to have occurred approximately 0.8 to 0.5 MYBP (= mid-Pleistocene). A modified calibration \( t = 19.7 \times 10^6D \) (Marten and Johnson 1986) using the same fossil evidence as Gutiérrez et al. (1983), but with corrected dating, yields the following estimates: 0.6 to 0.4 MYBP for the split of ruber and nuchalis from varius, and 3.9 to 2.8 MYBP for the split of these three taxa from thyroideus.

Estimates of divergence times based on mtDNA data can be compared with those obtained from nuclear (i.e. allozyme) data. The prevailing figure of 2% sequence divergence per million years (Brown et al. 1979, Shields and Wilson 1987) suggests earlier dates of divergence than those estimated by Nei’s D using the corrected calibration, that is, approximately 1.2 to 1.0 MYBP (=early Pleistocene) for the split of ruber and nuchalis from varius, and 5.1 to 4.9 MYBP (=early Pliocene) for divergence of the varius complex from thyroideus. Because the general applicability of this mtDNA clock to different taxonomic groups is controversial (see discussion by Avise 1994:103-106), we regard these estimates to be gross approximations at best. Nonetheless, both the allozyme and sequence data suggest that the varius group diverged from thyroideus during the Pliocene and that differentiation of ruber-nuchalis from varius occurred in the Pleistocene. As noted by Howell (1952), current zones of sympatry and limited hybridization between different forms of the varius complex probably reflect secondary contact after the last glaciation.

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