A Cytochrome-b Perspective on Passerina Bunting Relationships

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COLOR PLATE. The genus *Passerina*. The branching pattern reflects hypothesized relationships based on results of this study.
A CYTOCHROME-\textit{b} PERSPECTIVE ON PASSERINA BUNTING RELATIONSHIPS

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ABSTRACT.—We sequenced the complete mitochondrial cytochrome-\textit{b} gene (1,143 nucleotides) for representatives of each species in the cardinalid genera \textit{Passerina} (6 species), \textit{Guiraca} (1 species), and \textit{Cyanocompsa} (3 species), and used a variety of phylogenetic methods to address relationships within and among genera. We determined that \textit{Passerina}, as presently recognized, is paraphyletic. Lazuli Bunting (\textit{P. amoena}) is sister to the much larger Blue Grosbeak (\textit{Guiraca caerulea}). Indigo Bunting (\textit{P. cyanea}) and Lazuli Bunting are not sister taxa as generally thought. In all weighted parsimony trees and for the gamma-corrected HKY tree, Indigo Bunting is the sister of two sister groups, a “blue” (Lazuli Bunting and Blue Grosbeak) and a “painted” (Rosita’s Bunting [\textit{P. rositae}], Orange-breasted Bunting [\textit{P. leclancherii}], Varied Bunting [\textit{P. versicolor}], and Painted Bunting [\textit{P. ciris}] clade. The latter two species form a highly supported sister pair of relatively more recent origin. Uncorrected (\textit{p}) distances for ingroup (\textit{Passerina} and \textit{Guiraca}) taxa range from 3.0% (\textit{P. versicolor}–\textit{P. ciris}) to 7.6% (\textit{P. cyanea}–\textit{P. leclancherii}) and average 6.5% overall. Assuming a molecular clock, a bunting “radiation” between 4.1 and 7.3 Mya yielded four lineages. This timing is consistent with fossil evidence and coincides with a late-Miocene cooling during which a variety of western grassland habitats evolved. A reduction in size at that time may have allowed buntings to exploit that new food resource (grass seeds). We speculate that the Blue Grosbeak subsequently gained large size and widespread distribution as a result of ecological character displacement. Received 25 October 1999, accepted 16 September 2000.

THE BUNTINGS of the avian genus \textit{Passerina} (family Cardinalidae) include six species whose composite range includes Mexico, the United States, and parts of southern Canada. They are small, heavy-billed finches, most of which (see Thompson and Leu 1995) exhibit pronounced sexual dichromatism with brightly colored males and mostly brownish or olive females. Traditional museum studies relying upon comparisons of study skins (e.g. Ridgway 1901), as well as a more recent study employing numerical phenetic analyses of both skins and skeletons (Hellack and Schnell 1977), have concluded that the members of \textit{Passerina}, as presently recognized (Sibley and Monroe 1990, AOU 1998), form a natural group. Nevertheless, some authors (Phillips et al. 1964, Blake 1969, Mayr and Short 1970) place the monotypic Blue Grosbeak (\textit{Guiraca caerulea}) within this genus, and others (Paynter 1970) further expand \textit{Passerina} to include additional genera. Thus, despite the phenotypic similarities of the six recognized species, monophyly of \textit{Passerina} remains unresolved.

Relationships within \textit{Passerina} also remain unclear. Phillips et al. (1964) consider the Indigo Bunting (\textit{P. cyanea}) and the Lazuli Bunting (\textit{P. amoena}) conspecific. Hybridization on the Great Plains between \textit{P. cyanea} and \textit{P. amoena} has been well documented (Sibley and Short 1959, Emlen et al. 1975, Kroodsma et al. 1975). Similarly, the Painted Bunting (\textit{P. ciris}) and Varied Bunting (\textit{P. versicolor}) are known to have hybridized (Storer 1961). Whereas such hybridization might be taken as evidence of sister-species relationships, a known pairing between \textit{P. cyanea} and \textit{P. ciris} (Taylor 1974) muddles the picture. The ability to hybridize might be a mis-

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leading indicator of relationship (Zink and McKitrick 1995), therefore the construction of an independent phylogeny is warranted.

Our understanding of the species in *Passerina* is uneven. *Passerina cyanea* is among our best known songbirds with song, display behaviors, mating strategies, molt, and migration having been studied extensively (see Payne’s [1992] comprehensive review for references). At the other extreme are the endemic forms of southern Mexico, Leclancher’s (Orange-breasted) Bunting (*P. leclancherii*) and Rosita’s (Rose-bellied) Bunting (*P. rosita*), about which very little is known. A few comparative studies have involved most or all members of *Passerina*, examining molts and plumages (summarized in Thompson and Leu 1994), songs (Thompson 1968), and morphology (Hellack and Schnell 1977). However, interpretation of those studies is compromised by the lack of an explicit phylogenetic hypothesis (Brooks and McLennan 1991).

In this study, we used DNA sequences from the complete mitochondrial (mtDNA) protein coding cytochrome-*b* (cyt-*b*) gene to assess the monophyly of *Passerina* as presently recognized and to construct a phylogenetic hypothesis for the constituents of this group. Cyt-*b* and its flanking regions are well characterized in birds and its rate of evolution is particularly well suited for avian studies at the species level (Moore and DeFilippis 1997). The resulting phylogenetic tree provides a historical framework for a better understanding of the evolution of various life-history traits and for investigating historical biogeography.

**Methods**

*Taxon sampling.*—Outgroup choice is a critical component of molecular phylogenetic analysis (Smith 1994). Outgroups that are too distantly related to the group of interest lead to spurious rooting of the ingroup topology (Wheeler 1990). Relationships among Cardinalid genera are poorly understood, therefore an initial generic level phylogenetic survey of the group was performed. A cyt-*b* (430 bp) segment from at least one representative of each putative Cardinalid genus (excepting *Cyanocompsa*) was sequenced, as were representatives of several Thraupid genera, which were used as outgroups. The detailed results of that initial study are to be published elsewhere; however, in all phylogenetic analyses the *Passerina* buntings and *G. caerulea* were determined to constitute a clade (ingroup), sister to *Cyanocompsa* (outgroup). Those three genera were thus targeted for complete cyt-*b* gene sequencing. Because adding more taxa to the sister group increases the chance of obtaining the correct tree (Smith 1994), single representatives of all three members of *Cyanocompsa* were subsequently used. To obtain a measure of intraspecific genetic variation and to provide a check for potential sequencing errors, each of six species of *Passerina* and the monotypic *G. caerulea* (ingroup taxa) were represented by two different tissue specimens (Table 1).

**Laboratory protocols.**—Total DNA was extracted from fragments (~100 mg) of muscle or liver tissues following either a standard phenol/chloroform protocol (Hillis et al. 1996) or via incubation in Chelex/Proteinase K, a modification of Ellegren’s (1992) method. Overlapping sequence fragments were amplified via the polymerase chain reaction (PCR) using various combinations of the following published primers: L14851 (Kornegay et al. 1993), L14841 (Kocher et al. 1989), H15299 (Hackett 1996), H4A (Harshman 1996), LCBA, LCBB, LCBC, HCBC (Klicka et al. 1999); and two designed (J. Klicka) for general use on New World nine-primaried oscines: HCBRB (GGGAATGACTCGA YGTTTCA), and HCBJT (GGCTGGGGTGAAATTTTCTGGGTCT). A map of primer locations is available by request. PCR reactions were performed in 50 μL total volumes using a 1.5 mM concentration of MgCl₂, 1 μM concentrations of each primer, 0.2 mM final concentration of dNTPs, 0.5 μL of Tfl polymerase (Thermus flavus, Epicentre), 2.2 μL of 20× Tfl buffer (400 mM [NH₄]₂SO₄, and 1 M Tris-HCl [pH 9.0], Epicentre) and 10–1,000 ng (2 μL) of DNA template. A typical double-stranded amplification began with a 2 min denaturation at 94°C followed by 38 cycles of denaturing (94°C, 45 s), annealing (54°C, 1 min, 30 s), and primer extension (72°C, 1 min, 30 s) with a final extension of 72°C for 10 min. The presence of the desired product was verified by electrophoresis of 5 μL in a 1% agarose gel (Seakem LE, FMC) followed by Ethidium Bromide staining. Excess primers and dNTPs were then removed by enzymatic treatment of the PCR product with Exonuclease 1 and Shrimp Alkaline Phosphatase (U.S. Biochemical Corp.). To ensure sequencing accuracy, large fragment overlaps were used and wherever possible both light and heavy strands of the treated product were sequenced using either the original or nested primers. Sequencing reactions (Hillis et al. 1996) were done manually (product no. 70130, U.S.B.), run out on 6% acrylamide (Gene-Page, Amresco) gels, and visualized by autoradiography (³²P). Sequences (GenBank nos. AF301446–AF301462) were aligned visually using published chicken (*Gallus*; Desjardins and Morais 1990) and Snow Goose (*Chen [Anser] caerulescens*; Quinn and Wilson 1993) sequences for comparison.

**Data analysis.**—Parsimony (uniform and differential weighting schemes) and maximum-likelihood

TABLE 1. Species names, voucher numbers, and localities of origin of taxa sequenced, this study. UWBM = University of Washington Burke Museum; BMNH = J. F. Bell Museum of Natural History; LSUMNS = Louisiana State University Museum of Natural Science.

<table>
<thead>
<tr>
<th>Species</th>
<th>Museum</th>
<th>Number</th>
<th>Origin</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passerina cyanea 1</td>
<td>BMNH</td>
<td>X7250</td>
<td>USA, Wisconsin, St. Croix Co.</td>
<td>June 1994</td>
</tr>
<tr>
<td>Passerina cyanea 2</td>
<td>BMNH</td>
<td>JK94162</td>
<td>USA, Minnesota, Isanti Co.</td>
<td>5 August 1992</td>
</tr>
<tr>
<td>Passerina amoena 1</td>
<td>BMNH</td>
<td>JK95030</td>
<td>USA, Washington, Sherman Co., mouth of Deschutes River</td>
<td>29 June 1985</td>
</tr>
<tr>
<td>Passerina amoena 2</td>
<td>LSUMNS</td>
<td>B-4015</td>
<td>USA, Washington, Douglas Co., 5 mi N of Palisades</td>
<td>17 August 1987</td>
</tr>
<tr>
<td>Passerina ledancherii 1</td>
<td>UWBM</td>
<td>CWT034</td>
<td>Mexico, Chiapas</td>
<td>3 May 1993</td>
</tr>
<tr>
<td>Passerina ledancherii 2</td>
<td>UWBM</td>
<td>CWT040</td>
<td>Mexico, Chiapas</td>
<td>5 May 1993</td>
</tr>
<tr>
<td>Passerina rostae 1</td>
<td>UWBM</td>
<td>CWT033</td>
<td>Mexico, Chiapas, Monte Bonito</td>
<td>2 May 1993</td>
</tr>
<tr>
<td>Passerina rostae 2</td>
<td>UWBM</td>
<td>CWT036</td>
<td>Mexico, Chiapas</td>
<td>4 May 1993</td>
</tr>
<tr>
<td>Passerina ciris 1</td>
<td>LSUMNS</td>
<td>B-5693</td>
<td>USA, Louisiana, Cameron Parish, Monkey Island, near Cameron</td>
<td>29 April 1984</td>
</tr>
<tr>
<td>Passerina ciris 2</td>
<td>LSUMNS</td>
<td>B-5694</td>
<td>USA, Louisiana, Cameron Parish, Monkey Island, near Cameron</td>
<td>29 April 1984</td>
</tr>
<tr>
<td>Poserina versicolor 1</td>
<td>UWBM</td>
<td>CWT097</td>
<td>Mexico, Coahuila, 35 km N of Saltillo</td>
<td>24 May 1993</td>
</tr>
<tr>
<td>Poserina versicolor 2</td>
<td>UWBM</td>
<td>CWT097</td>
<td>Mexico, Coahuila, 35 km N of Saltillo</td>
<td>24 May 1993</td>
</tr>
<tr>
<td>Guiraca caerulea 1</td>
<td>LSUMNS</td>
<td>B-21812</td>
<td>USA, Texas, Jeff Davis Co., Davis Mountains State Park</td>
<td>7 September 1992</td>
</tr>
<tr>
<td>Guiraca caerulea 2</td>
<td>LSUMNS</td>
<td>B-20958</td>
<td>USA, Louisiana, Cameron Parish, Garner Ridge</td>
<td>12 November 1992</td>
</tr>
<tr>
<td>Cyanocompsa brissonii</td>
<td>LSUMNS</td>
<td>B-18658</td>
<td>Bolivia, Dept. Santa Cruz, Cordellara</td>
<td>8 September 1990</td>
</tr>
<tr>
<td>Cyanocompsa cyanades</td>
<td>LSUMNS</td>
<td>B-12708</td>
<td>Bolivia, Dept. Santa Cruz, Velasco</td>
<td>27 July 1988</td>
</tr>
<tr>
<td>Cyanocompsa parellina</td>
<td>UWBM</td>
<td>CWT082</td>
<td>Mexico, Oaxaca, Chiwela, 6 mi NW</td>
<td>21 May 1993</td>
</tr>
</tbody>
</table>
Table 2. Cytochrome-b substitution type proportions shown above the diagonal; corresponding six-parameter weights shown below. Values were obtained by reconstructing the data onto the shortest equally weighted tree. Corrections for triangle inequality shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>11.6%</td>
<td>23.5%</td>
<td>4.2%</td>
</tr>
<tr>
<td>C</td>
<td>4.1</td>
<td></td>
<td>1.0%</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2.8</td>
<td>8.8</td>
<td>(6.9)</td>
<td>0.4%</td>
</tr>
<tr>
<td>T</td>
<td>(5.1)</td>
<td>1.0</td>
<td>10.6</td>
<td>(7.9)</td>
</tr>
</tbody>
</table>

Mutation rates are known to vary throughout the cyt-b gene, presumably due to functional constraints at the amino acid level (e.g. Howell 1989, Krajewski and King 1996, Griffiths 1997). We addressed that problem by using MP and a different weighting scheme in which transitions located in putatively more variable regions were down-weighted. Three rate classes of residues were defined using Howell’s (1989) structural model for mouse cyt-b in which individual residues were classified as evolutionarily conserved, intermediate, or variable. Although similar, these partitions do not correspond exactly to those used in other published studies (e.g. Griffiths 1997). We performed an analysis in which the sequence constituting each of these three partitions was weighted according to the inherent transition:transversion ratio (calculated in MacClade as described above) in that data partition. The result was transversions weighted 3.0:1, 6.2:1, and 4.8:1 over transitions for conserved, intermediate, and variable sequence partitions respectively. Standard tree statistics, including length, consistency index (CI), retention index (RI), and rescaled consistency index (RC) were generated for all parsimony reconstructions.

All maximum-likelihood (ML) analyses used empirical base frequencies, with full heuristic searches. An initial analysis was based on Felsenstein’s (1981, [F81]) model of DNA evolution which corrects for unequal base frequencies. The data were subsequently analyzed with the HKY85 (Hasegawa et al. 1985) model which additionally accommodates transition biases. That model in particular has proven to be effective in describing the dynamics of many genes (Goldman 1993, Yang 1996). This analysis used a transversion transition ratio of 6.9 and a gamma distribution with a shape parameter (α) of 0.106. These parameters were estimated using the stable (1:1, 2:1) MP trees following the recommendation of Swofford et al. (1996). Because the models used are nested, we were able to assess statistically their relative values by comparing log-likelihood values using likelihood ratio tests (LRT, Huelsenbeck and Rannala 1997).

Node support for MP analyses was determined via bootstrapping (Felsenstein 1985) with 500 replications using full heuristic searches and random addition of taxa. One hundred such replicates were done for ML analyses. Decay indices (Bremer 1994) were generated for the most highly supported parsimony-derived topology using TREEROT (Sorenson 1996). MacClade (Maddison and Maddison 1992) was used to compare tree lengths for competing tree topologies. Alternative topologies were tested using the Kishino-Hasegawa (1989) maximum-likelihood ratio test.

Biogeographic interpretations are enhanced by invoking a molecular clock. The validity of assuming clock-like rates of evolution for those taxa was tested via a likelihood-ratio test (Huelsenbeck and Rannala 1997) by comparing log-likelihood values from ML trees constructed with and without a molecular clock constraint using the HKY85/gamma(1) correct model of sequence evolution. To allow direct comparison with the Fleischer et al. (1998) songbird-specific clock calibration, we generated a matrix of K2P F-corrected pairwise distances following their protocol. The α value used (0.300) was estimated via the method of Sullivan et al. (1995) in PAUP* from the shortest unweighted MP tree.

Results

Sequence variation.—All 1,143 nucleotides were aligned with no insertions, deletions, or nonsense codons in evidence. Although the amplification of nuclear pseudogenes of mitochondrial origin can confound phylogenetic analysis (Zhang and Hewitt 1996), a number of factors make it unlikely that our sequences are nuclear copies. In addition to straightforward alignment and lack of insertions or deletions, heme-ligating histidines and other conserved residues (Howell 1989) were identified, and no coamplified DNA product (i.e. ambiguous sites) was apparent on the sequencing gels. For birds, nuclear copies are most often associated with the use of blood as a DNA source (Johnson and Sorenson 1998); we used muscle or liver tissue exclusively. Furthermore, the nucleotide bias observed in gene partitions of this data set is consistent with that reported for other birds (Moore and DeFilippis 1997). Lastly, our results are reasonable from both a biological and geographical perspective, as we show below.

Of the 1,143 aligned sites, 240 were variable, and of those, 195 were potentially phylogenetically informative (Table 3). Among those sites, 164 (84%) were at third positions, 28 (14.5%) at first positions, and only 3 (1.5%) at second position sites. Relative rate calculations indicate
that third-position sites are evolving 40\times faster than are second-position sites. Nucleotide compositional bias was most acute at third codon positions (0.173), followed by second (0.058) and first (0.006) positions. That was due mainly to the expected strong bias against G (3.8%) at that site. Overall, base composition (Table 3) and base-composition biases are similar to those recovered in other avian cyt-b studies (e.g. Kornegay et al. 1993, Nunn et al. 1996).

Most empirical gamma-shape parameter estimates (\(\alpha\)) vary from 0.1 to 0.5 (Yang 1996) and the composite sequence estimate for those data falls within this range. However, \(\alpha\) estimates for first and third positions differ by two orders of magnitude (0.007 vs. 1.07), suggesting that severe among-site rate heterogeneity exists at first positions, whereas the higher value associated with third positions suggests a more even distribution of substitutions. Despite the fact that the preponderance of phylogenetically informative sites are at third positions, we note that the overall \(\alpha\) estimate (0.106) imposed differs from the estimate for only third positions by an order of magnitude.

Uncorrected percentage sequence divergence (\(p\), Table 4) between *Passerina* species ranges from 3.0\% (P. versicolor–P. ciris) to 7.6\% (P. cyanae–P. leclancherii) and averages 6.5 (±1.0\%, \(n = 84\)). Minimum within-species pairwise values range from 0.1\% (P. ciris) to 0.4\% (P. cyanae and G. caerulea). Overall, intraspecific divergence was 0.24 (±0.1\%, \(n = 7\)), less than the empirical value of 0.7\% that was calculated for a range of avian taxa (Moore 1995). Despite the relatively low divergence values, saturation plots for the combined dataset (*Passerina*, G. caerulea, and Cyanocompsa) provide evidence that multiple substitutions have begun to accumulate in third position transitions (Fig. 1, other [linear] plots not shown).

For these data, log-likelihoods derived from analyses with and without the assumption of a molecular clock (Table 5) did not differ significantly (0.5 > \(P > 0.1\)) suggesting that these taxa display an approximately uniform rate of substitution such that inference of divergence times is appropriate.

**Phylogenetic analyses.**—From the many analyses performed, two fully resolved topologies were consistently recovered (Fig. 2A, C). They differ only in the placement of *P. leclancherii*. Equally weighted parsimony, distance, and the F81 maximum-likelihood analysis yield a topology in which the Mexican endemics *P. leclancherii* and *P. rositae* are sisters (Fig. 2A), whereas in all weighted parsimony and other likelihood analyses, *P. leclancherii* is sister to the well supported *P. ciris–P. versicolor* clade (Fig. 2C). With the data weighted equally, those topologies differ by a single step (Fig. 2A: length \([l] = 419, CI = 0.649, RI = 0.742, RC = 0.481\); 3c:1 = 420, CI = 0.648, RI = 0.740, RC = 0.479; Kishino-Hasegawa test [1989], \(P = 0.3950\)). Because the Figure 2C topology was recovered in all weighted-parsimony analyses and in the best fit maximum-likelihood analysis, we consider this our best estimate of relationships within that group. Lanyon (1993) advises that systematists identify both a "best estimate" and [italics his] a "reliable estimate" of phylogenetic relationships. In that spirit, we consider the more conservative, bootstrapped equally weighted MP tree (Fig. 2B) as our most reliable estimate.

In both fully resolved trees, *P. cyanae* is sister to all other ingroup taxa, although that node is poorly supported in several analyses. Two main clades are supported in both trees, a predominately temperate zone ("blue" = *G. caerulea* + *P. amoena*) clade and a more sedentary group ("painted" = *P. rositae* + *P. leclancherii* +

---

**Table 3.** Overall and codon position-specific dynamics of the cytochrome-b gene for all taxa. Mean base composition is averaged over all sequences using PAUP*. Transition-transversion ratio (Ts/Tv) values are the average number of changes reconstructed on the weighted parsimony topology.

<table>
<thead>
<tr>
<th>Position</th>
<th>Number of sites</th>
<th>Variable sites</th>
<th>Parsimony informative</th>
<th>Relative rate</th>
<th>%A</th>
<th>%C</th>
<th>%G</th>
<th>%T</th>
<th>Ts/Tv</th>
<th>(\alpha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>1143</td>
<td>240</td>
<td>195</td>
<td>16.0</td>
<td>27.3</td>
<td>34.7</td>
<td>13.3</td>
<td>24.7</td>
<td>5.08</td>
<td>0.106</td>
</tr>
<tr>
<td>1st</td>
<td>381</td>
<td>33</td>
<td>28</td>
<td>6.6</td>
<td>25.6</td>
<td>30.0</td>
<td>23.4</td>
<td>21.0</td>
<td>8.60</td>
<td>0.007</td>
</tr>
<tr>
<td>2nd</td>
<td>381</td>
<td>5</td>
<td>3</td>
<td>1.0</td>
<td>20.7</td>
<td>25.4</td>
<td>12.6</td>
<td>41.3</td>
<td>1.50</td>
<td>(\approx)</td>
</tr>
<tr>
<td>3rd</td>
<td>381</td>
<td>202</td>
<td>164</td>
<td>40.4</td>
<td>35.5</td>
<td>48.8</td>
<td>3.8</td>
<td>11.9</td>
<td>4.91</td>
<td>1.07</td>
</tr>
</tbody>
</table>
Table 4. Observed overall genetic pairwise distances for complete cytochrome-\(b\) gene. Values above the diagonal are Kimura two-parameter genetic distances corrected for among-site rate heterogeneity; uncorrected (\(p\)) sequence divergences below the diagonal.

\[
\begin{array}{cccccccccccccccc}
\text{Species} & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12 & 13 & 14 & 15 & 16 & 17 \\
1 P. leclancherii & 0.044 & 0.091 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 & 0.088 \\
2 P. cyanea & 0.044 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 & 0.036 \\
3 P. amoena & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 & 0.035 \\
4 G. caerulea & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 & 0.034 \\
5 Passerina & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 & 0.033 \\
6 P. rositae & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 & 0.031 \\
7 P. versicolor & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 & 0.030 \\
8 P. ciris & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 & 0.029 \\
9 P. cyanea & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 & 0.028 \\
10 P. versicolor & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 & 0.027 \\
11 P. rositae & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 & 0.026 \\
12 P. versicolor & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 & 0.025 \\
13 P. ciris & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 & 0.024 \\
14 P. cyanea & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 & 0.023 \\
15 P. versicolor & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 & 0.022 \\
16 P. ciris & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 & 0.021 \\
17 P. cyanea & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 & 0.020 \\
\end{array}
\]

FIG. 1. Saturation plot for the cytochrome-\(b\) gene, third-position transitions only. Within-species comparisons are included. Similar plots of other codon positions and transformation types (not shown) were approximately linear.

\(P. \text{versicolor} + P. \text{ciris}\) centered in the aridlands of the southwestern United States and western Mexico. Levels of support as measured by decay indices (Fig. 2A) and bootstrap values vary among nodes (Table 5) and between analytical methods. Perhaps the most extreme case of the latter is the node which unites the painted clade (C on Fig. 2C). Under the HKY\(^T\) model, that node has 78% bootstrap support yet its counterpart on the most parsimonious equal-weights topology has a decay index of 1 and <50% bootstrap support. Similarly, support for a basal \(P. \text{cyanea}\) (node B) is moderately high under the equal and 2:1 parsimony analysis but is lacking under the HKY\(^T\) maximum likelihood model.

Both trees demonstrate that the monotypic \(G. \text{caerulea}\), despite having a mass approximately twice that of most \(Passerina\) buntings, is embedded within \(Passerina\), rendering the genus paraphyletic. In fact, in all analyses \(G. \text{caerulea}\) and \(P. \text{amoena}\) were sister taxa with relatively high support from both bootstrap and decay values (Table 5). Constraining \(P. \text{cyanea}\) and \(P. \text{amoena}\) to be sisters (the traditional arrangement) with \(G. \text{caerulea}\) sister to all of \(Passerina\), requires 17 additional (equal weight) steps and results in a significantly worse topology (Kishino-Hasegawa [1989] test, \(P = 0.0498\)). Likewise, forcing \(G. \text{caerulea}\) to be sister to the similarly sized spe-
TABLE 5. Levels of bootstrap support for select nodes (see Fig. 2c) for combined data using maximum parsimony and maximum likelihood analyses. All MP (×500) and ML (×100) replicates included full heuristic searches. For ML estimates, empirical nucleotide frequencies were used and \( \alpha (=0.106) \) and \( T_s/T_v (=6.92) \) were estimated from the data under the HKY model of evolution.

<table>
<thead>
<tr>
<th>Node</th>
<th>Parsimony</th>
<th>Likelihood</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>71</td>
<td>74</td>
</tr>
<tr>
<td>C</td>
<td>52</td>
<td>63</td>
</tr>
<tr>
<td>D</td>
<td>52</td>
<td>63</td>
</tr>
<tr>
<td>E</td>
<td>96</td>
<td>95</td>
</tr>
<tr>
<td>F</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
<td>73</td>
<td>69</td>
</tr>
</tbody>
</table>

- For MP, \( \times 9.5 = \) native rate weighting, \( Y = \) (Yoder) \( \times 5 \) weighting of third position transversions only, \( 6P = \) six parameter weighting, \( \text{CS} = \) codon specific weighting, \( \text{How} = \) weighted according to Howell's (1989) cyt-b variability categories (see text).

LRTs indicate that HKY is a significant improvement over F81 (null hypothesis: transition rate equals transversion rate is rejected \([-2 \log D = 455.4, df = 1, P < 0.001]\)); HkyT is significantly better than uncorrected HKY (null hypothesis: equal rates among sites is rejected \([-2 \log D = 376.4, df = 1, P < 0.001]\)). LRT for molecular clock is not significantly different \([-2 \log D = 16, df = 15, 0.5 < P < 0.01]\). Unconstrained ML = 3,984.3.

**FIG. 2.** Results of analyses. (A) Topology derived from equally weighted MP analysis; decay indices are shown above nodes. Branch lengths shown are proportional to genetic distances derived from an I-corrected (\( \alpha = 0.106 \)) Tamura-Nei (1993) neighbor-joining algorithm. (B) bootstrapped (×500) equal weights MP tree. (C) Bootstrapped (×100) ML tree with best likelihood score (HkyT); \( \alpha = 0.106, T_s/T_v = 6.93 \). Lettered nodes refer to Table 5.
cies of Cyanocompsa yields an even worse tree (33 steps, \( P = 0.0023 \)). Thus, the mitochondrial gene tree is unambiguous regarding the placement of \( G.\ caerulea \). The other strongly supported terminal taxon pair is \( P.\ versicolor-P.\ ciris \), receiving no less than 97% bootstrap support in any analysis.

**DISCUSSION**

*Passerina paraphyly.*—A surprising finding of this study was the high bootstrap support across all analyses (average of 96.5%) for a \( G.\ caerulea-P.\ amoena \) sister relationship, demonstrating that \( G.\ caerulea \) is derived from within the *Passerina* assemblage. This result indicates that a merging of the monotypic genus Guiraca into *Passerina* is warranted. The conventional “‘superspecies’” groupings of \( P.\ cyanae-P.\ amoena \) with \( P.\ versicolor \) (Mengel 1970) or \( P.\ caerulea-P.\ amoena \) with \( P.\ versicolor \) and \( P.\ ciris \) (Mayr and Short 1970) are rejected by our data. Those workers who, on the basis of morphological and behavioral evidence, had considered \( G.\ caerulea \) to be a member of the genus *Passerina* (despite its much larger size; e.g. Phillips et al. 1964, Blake 1969, Mayr and Short 1970) are vindicated; although, we know of no one who hypothesized a \( G.\ caerulea-P.\ amoena \) sister relationship. Our result conflicts with conclusions drawn from a family-level allozyme study (Tamplin et al. 1993). Those authors concluded that \( G.\ caerulea \) might be more closely related to *Pheucticus* than to either *Passerina* or *Cyanocompsa*; however, they noted that the strong plumage and vocal similarities between \( G.\ caerulea \) and the *Passerina* buntings, seem unlikely to be merely shared primitive characters. Indeed, their (Tamplin et al. 1993; Fig. 2) consensus of 172 most parsimonious trees does not support a *Guiraca–Pheucticus* relationship. Although mtDNA and allozyme surveys are often congruent at the intrageneric level, the apparent lack of resolution in the Tamplin et al. (1993) study may reflect a taxon sampling problem or the limited resolving power of allozymes for higher (intergeneric) taxonomic levels.

We suggest that if \( G.\ caerulea \) were the size of the other buntings (adult males, approximately 15–20 g, see below), it would have long been classified as a member of *Passerina*, perhaps even as sister to \( P.\ amoena \). Study-skin comparisons provided additional evidence in support of that relationship. Among members of this genus, only adult males of \( P.\ amoena \) and \( G.\ caerulea \) have conspicuous, complete wingbars in definitive plumage, and only in the plumage of this pair does a cinnamon (closest to Mikado Brown [no. 121C], of Smite 1975) color occur (similar to that of *Cyanocompsa* females). Black streaking dorsally is also most conspicuous in this pair although it occurs to a lesser degree in \( P.\ cyanae \). *Guiraca caerulea* (nearest Small Blue [no. 70], Smite 1975) and \( P.\ amoena \) (nearest Turquoise Blue [no. 65], Smite 1975) are rather different shades of blue. Curiously, both of those shades occur in \( P.\ cyanae \), the blue of \( G.\ caerulea \) occurring on the head and throat and the blue of \( P.\ amoena \) occurring on the lower body (rump).

The “‘painted’” clade (node C, Fig. 2C) is united similarly by plumage characteristics. All have some blue plumage, but it is the presence of patches of bright carotenoid pigments (yellow, pink, scarlet) and conspicuous eye rings that sets them apart. It is selection on precisely these sorts of sexually and socially selected traits that is presumed to lead to accelerated divergence (West-Eberhard 1983, Zink 1996), speciation, and ultimately taxonomic diversity. The short internode distances within the “‘painted’” clade (e.g. node D, Fig. 2A, C) is consistent with that interpretation, that is, closely spaced speciation events.

**Ecological implications.**—The *Passerina* buntings (exclusive of \( G.\ caerulea \)) possess some of the smaller body sizes in the Cardinalidae, as shown by the morphometric phenetic analyses (PCA plots) of that group (Hellack and Schnell 1977, Tamplin et al. 1993). With the addition of \( G.\ caerulea \), *Passerina* becomes composed of two discrete size classes (“‘bunting’” and “‘grosbeak’,” see Fig. 3). Notably, \( P.\ amoena \) and \( G.\ caerulea \) are closely related sister species, yet the mass of the former is approximately one-half that of the latter. A comparable size dichotomy is apparent within the sister genus *Cyanocompsa* where \( C.\ parellina \) (15.9 ± 1.23 g, \( n = 19 \)) and \( C.\ cyanoideus \) (35.8 ± 2.77 g, \( n = 13 \)) also exhibit a bunting versus grosbeak size relationship whereas a third *Cyanocompsa* member, *brissonii*, is reported of an intermediate size. Collectively, these observations suggest that body size (mass) is not a particularly conservative trait within this assemblage of birds.
Approximate breeding distributions and "sizes" of all Passerina members shown in a phylogenetic context. Note that the distribution of Passerina rositae is restricted to the Pacific Slope of the Isthmus of Tehuantepec. Silhouette sizes reflect relative average mass, obtained from museum labels of adult male specimens: P. cyanea (14.8 ± 0.8 g, n = 31); P. amoena (16.8 ± 2.23 g, n = 4); P. rositae (20.07 ± 0.16 g, n = 61); P. leclancherii (13.70 ± 0.19 g, n = 105); P. versicolor (13.0 ± 1.78 g, n = 104); P. ciris (16.47 ± 2.11 g, n = 9); and G. caerulea (32.6 ± 2.87 g, n = 15).

The phylogeny shown (Fig. 2C) indicates that "bunting" size is the ancestral state for Passerina with G. caerulea secondarily acquiring a "grosbeak" size. Similarities in the size and shape of Passerina buntings, taken together with their relative lack of range overlap (Fig. 3), suggest the possibility that many, or all, are "ecological equivalents" and by extension, competitors for resources. A composite map of all bunting breeding distributions (not shown) indicates that virtually all suitable bunting habitat throughout North America is occupied by one bunting species or another. The relatively huge and overlapping range of G. caerulea with respect to those of the smaller buntings (Fig. 3) is likely a consequence of its now dissimilar size and food resource needs. We do not know whether that morphological shift was the direct result of competition with congeners (ecological character displacement) or if it occurred in response to alternative selection pressures. Character displacement as a result of interspecific competition is difficult to demonstrate (Schluter et al. 1985) but may be tested by correlating shifts in morphology with degree of sympathy (or allopatry) with competitors. Guiraca caerulea, with seven described subspecies (Storer and Zimmerman 1959), varies in size across its range and should be studied in that context. A hypothesis of character displacement predicts that the largest G. caerulea should occur where the species is sympatric with high Passerina densities and the smallest in regions unoccupied (or sparsely populated) by any Passerina species.

Little is known about intrageneric competitive interactions for this group. Examination of breeding-bird survey maps (Price et al. 1995) suggests that actual overlaps in breeding distribution may be less extensive than that typically depicted in maps of breeding distributions (such as those shown, Fig. 3), and where overlap does occur, little is known about partitioning of habitat and food resources. In areas of overlap on the western Great Plains, the similarly sized but nonsister species P. cyanea and P. amoena defend interspecific territories (Emlen et al. 1975). Interspecific territoriality does not occur between the more broadly overlapping pair P. cyanea and P. ciris (Forsythe 1974), suggesting perhaps a diminution of competitive interactions over time and phylogenetic distance. Also, P. cyanea does not defend territories against the visually similar but much larger G. caerulea (Payne 1992). Indeed, the emphasis on breeding distributions may be overstated; competitive interactions between species of Passerina might be best understood by a winter study in southwestern Mexico where six of the seven taxa occur in sympatry.

Approximate times of divergence.—A temporal interpretation of the phylogeny requires a roughly uniform substitution rate among taxa. The likelihood-ratio test for molecular clock indicates base substitutions are accumulating in a sufficiently clock-like manner to allow such interpretations. We acknowledge that application of a molecular clock remains controversial (e.g. Hillis et al. 1996, Klicka and Zink 1997) but we are also aware of the heuristic value of assigning tentative divergence dates to phyloge-
netic branching events. We use two independently derived clock calibrations. The commonly used rate of 2% divergence per million years is the rate obtained by independent fossil calibrations from an array of avian orders (Klicka and Zink 1997). That this same rate is also derived for mammals (Wilson et al. 1985) and arthropods (Brower 1994) lends some credibility to its generality. Because variation in evolutionary rates has been shown to be related to variation in body size and its correlates (e.g. Martin and Zink 1997), that this same rate is also derived from an array of avian orders (Klicka and Zink 1998), a 1.6% rate using corrected (K2-P) distances (Table 4).

These rates suggest that Cyanocompsa and Passerina buntings diverged from a common ancestor approximately 4.1 to 7.3 Mya. This coincides with a Late Miocene period of accelerated cooling and drying during which forests and woodlands gave way to an increasing variety of grassland habitats in western North America (Riddle 1995 and references therein). A reduction in size and an increased ability to exploit grassland food resources may have played a role in a Passerina radiation (diversification and widespread distribution) at that time. This time frame is supported by a Passerina fossil (Steadman and McKitrick 1982) from Chihuahua, Mexico, dated to ~4 Mya. The fossil fragments identify an extinct Passerina species that was intermediate between P. amoena and G. caerulea both qualitatively and in size, and from a region where both presently occur.

Among the other well-resolved nodes, the calibrations indicate that G. caerulea–P. amoena diverged approximately 2.4 to 3.7 Mya and the most recent split within the group, P. ciris–P. versicolor, occurred 1.5 to 2.1 Mya. The bunting ‘pair’ P. amoena–P. cyanea has figured prominently (see Sibley and Short 1959) in the development of evolutionary models of songbird evolution. Typically, those (e.g. Mengel 1970, Hubbard 1973) invoke recent glacial advances (i.e. the last 250,000 years) that reconfigure habitats in such a way that populations fragment and subsequently speciate while in isolation. This ‘Late Pleistocene Origins’ model was rejected using molecular data (Klicka and Zink 1997, 1998), a conclusion supported by this study. Here we show that P. amoena–P. cyanea are not even sister taxa and that it is unlikely that any members of this genus originated as a consequence of Late Pleistocene glaciations.

Collectively, the lack of consistent bootstrap support at nodes B, D, and E (Fig. 2C) suggest a burst of simultaneous speciation among four Passerina lineages, with the P. amoena–G. caerulea and P. versicolor–P. ciris lines splitting again at some later time. Zink et al. (1998) note that ‘star phylogenies’ such as this might also be arrived at via bursts of extinction. Alternatively, additional data may change that interpretation entirely by strengthening the nodes in question. We are currently investigating that possibility by sequencing additional gene regions (ND6 and control region) for all Cyanocompsa and Passerina taxa. A thorough biogeographic assessment requires the teasing apart of these alternatives.

Hybridization as a measure of relationship.—Because a well-documented and extensive hybrid zone exists where they are sympatric on the Great Plains (Sibley and Short 1959, Emlen et al. 1975, Kroodsma 1975), P. amoena and P. cyanea have been treated as conspecifics by some (e.g. Phillips et al. 1964) and considered a ‘superspecies’ of very recent origin by others (Mayr and Short 1970). A single hybrid specimen is known for another partly sympatric bunting sister pair, P. ciris and P. versicolor (Storer 1961). The inference drawn from that observation is that P. amoena and P. cyanea are the more closely related of those pairs (e.g. Storer 1961). The results of this study challenge that interpretation. Passerina amoena and P. cyanea are not sister species, whereas P. ciris and P. versicolor have the most recent origins of any extant members of the genus. It is becoming increasingly clear that for birds, the ability to hybridize and the degree to which hybridization occurs are not necessarily good measures of relatedness (Prager and Wilson 1975). We add P. amoena–P. cyanea to the growing list of nonsister taxa that not only retain the ancestral capacity to hybridize, but also to form an ap-
parent hybrid swarm (e.g., *Colaptes*, Moore et al. 1991; *Icterus*, Freeman and Zink 1995).

ACKNOWLEDGMENTS

This paper is dedicated to the life and memory of Allan Galante who loved Mexico, its land, people, culture, music, and natural history, including its bird life. C.W.T. is grateful for financial and logistical support from (1) the Eddy Endowment for Excellence in Ornithology, (2) C. Wood, C. Spaw, and the Rohwer Lab Group at the Burke Museum, University of Washington, (3) two Chicago Zoological Society Society Conservation and Research Fund awards, (4) a Center for the Study of Tropical Birds award, (5) Adolfo Navarro and Octavio Rojas at the Museo de Zoologia at the Universidad Nacional Autonoma de Mexico, (6) Eduardo Morales and Eduardo Espinosa of the Instituto de Historia Natural, Gobierno de Chiapas, Mexico, and (7) the Western Foundation of Vertebrate Zoology. We thank the curators and staff at the Louisiana State University Museum of Zoology and the University of Washington Burke Museum for acquiring and loaning tissue specimens. A. Phillips provided valuable field assistance as did T. Brashear. J.K. acknowledges funding support from the Dayton and Wilkie Funds of the J. F. Bell Museum of Natural History. Funding for laboratory work was provided by R.M.Z.’s NSF grant DEB-9317945.

LITERATURE CITED


Associate Editor: R. Prum