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NEW WORLD NINE-PRIMARIED OSCINE RELATIONSHIPS: CONSTRUCTING A MITOCHONDRIAL DNA FRAMEWORK

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ABSTRACT.—Historically, a paucity of comparative morphological characters has led to much debate regarding relationships within and among the major lineages of New World nine-primaried oscines. More recently, DNA-DNA hybridization studies have provided novel and testable hypotheses of relationships, although no consensus has been reached. For 40 songbird taxa, we obtained 1,929 base pairs (bp) of DNA sequence from the mitochondrial cytochrome-*b* (894 bp) and NADH dehydrogenase subunit 2 (1,035 bp) genes. Phylogenetic analyses confirm the monophyly of this assemblage as traditionally defined. The lineages delineated historically on morphological grounds are retained; finches (Fringillinae) are sister to a well-supported clade (Emberizinae) containing blackbirds (Icterini), sparrows (Emberizini), wood-warblers (Parulini), tanagers (Thraupini), and cardinal-grosbeaks (Cardinalini). However, each tribe individually is either paraphyletic or polyphyletic with respect to most recent songbird classifications. Our results suggest that *Euphonia* is not a tanager but perhaps represents a derived form of cardueline finch. *Piranga*, traditionally considered a typical tanager, is a cardinaline in all of our analyses. *Calcarius* falls outside the sparrow lineage in all of our analyses, but its true affinities remain unclear. Elements of four different AOU families are represented in our clade Thraupini. The inclusion of several "tanager-finches" (*Haplospiza, Diglossa, Tiaris, Volatinia, Sporophila*) and a nectarivore (*Coereba*) in this clade is consistent with findings from other molecular phylogenies in suggesting that convergence in feeding specializations among some lineages has confounded traditional morphological classifications. We obtained a novel arrangement of relationships among tribes in our "best" topology; Cardinalini is sister to the rest of the Emberizinae assemblage (as defined by Sibley and Ahlquist [1990]), and Thraupini is sister to a clade containing Icterini, Emberizini, and Parulini. Despite nearly 2,000 bp of sequence for each taxon, and a high degree of stability across most weighting schemes and analytical methods, most nodes lack strong bootstrap support. The ND2 gene provided higher resolution than did cytochrome *b*, but combining genes provided the most highly supported and resolved topology. We consider the phylogeny a working hypothesis to be used as a guide for further studies within the nine-primaried oscine assemblage. *Received 6 November 1998, accepted 4 August 1999.*

THE NEW WORLD nine-primaried oscines, with about 1,000 species, represent roughly 10% of all living species of birds. This group is "by almost unanimous agreement, an assemblage of families of close relationship and common ancestry" (Tordoff 1954a:274). Although this statement still rings true, the systematic relationships within and between the major groups (i.e. families of AOU [1998], tribes of Sibley and Monroe [1990]; unless otherwise specified we use the nomenclature of the latter throughout this paper) of nine-primaried oscines continue to be among the most problematic systematic questions within an avian order.

A long history of attempts to clarify relationships among members of this assemblage has included comparative studies of external morphology (Ridgway 1901, 1902), jaw musculature (Beecher 1953), pelvic musculature and serology (Stallcup 1954), cranial and palatal characters (Tordoff 1954b), and appendicular myology (Raikow 1978). Such works have led to a general agreement regarding the taxonomic boundaries of this passerine assemblage, but they have yielded only a handful of useful characters with which to define the relationships among the component groups (Mayr and Amadon 1951, Sibley 1970, Feduccia 1996). Of these characters, many are often inconsistent with one another (Bledsoe 1988). This result has led to a series of linear classifications (see Sibley and Ahlquist [1990] for a comprehensive re-

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view) that differ only slightly from one another. The traditional view of nine-primaried oscine evolution held that "the major groups are products of adaptive radiations into discrete adaptive zones defined mainly in terms of feeding specializations" (Raikow 1978:34). Early classifications based on this perspective had in common the following six "core" groups: the cardueline finches (Sushkin 1925), wood-warblers, blackbirds, sparrows, cardinal-grosbeaks, and tanagers. However, overlapping bill morphologies between the latter three have led to relatively arbitrary boundary distinctions (e.g. Sclater 1886, Ridgway 1902, Tordoff 1954b). As a consequence, genera with "finch type" bills have been shuffled about taxonomically as have a few other genera (e.g. *Cyanerpes, Spiza, Icteria*) that failed to conform to one of the general adaptive (trophic) types. Because of the extreme morphological uniformity (i.e. paucity of phylogenetically informative characters), earlier oscine classifications lacked insight into among-group relationships, and with the exception of Raikow's (1978) cladistic analysis, few offer much in the way of testable phylogenetic hypotheses. Classifications based on a new source of data, DNA-DNA hybridization comparisons (Bledsoe 1988, Sibley and Ahlquist 1990), have provided a fresh perspective that has challenged traditional views by demonstrating that the adaptive radiation of New World nine-primaried oscines is characterized by convergence at many levels. These molecular phylogenies have provided novel insights concerning the phylogenetic placement of difficult (anomalous) genera and also furnished testable hypotheses of relationships among groups. More recently, the direct sequencing of mitochondrial DNA (mtDNA) has provided an abundance of characters for the construction of phylogenetic hypotheses (e.g. see Mindell 1997).

Despite much prior debate, it is now generally accepted (i.e. the prevailing phylogenetic hypotheses suggest) that the New World nine-primaried oscines (the Fringillidae of Sibley and Ahlquist [1990]) are comprised of two main sister clades, one containing the fringilline and cardueline finches along with the Hawaiian honeycreepers (subfamily Fringillinae), and the other (subfamily Emberizinae) consisting of the tribes Emberizini (true buntings and New World sparrows), Parulini (wood-warblers), Thraupini (tanagers and tanager-finches), Cardinalini (cardinal-grosbeaks), and Icterini (blackbirds and allies). Because Fringillinae is considered primarily an Old World group, and most members of the Emberizinae are thought to be of New World origin, the latter is the more narrowly defined "New World nine-primaried oscines" of some authors (e.g. Sibley and Ahlquist 1990). Because neither subfamily is strictly Old or New World in distribution, we favor the traditional and more inclusive definition of this term. The monotypic genus *Peucedramus*, long considered a member of Parulini, has been excluded from that group on both morphological (George 1962) and biochemical grounds (Sibley and Ahlquist 1990) and is tentatively considered the sister taxon (subfamily Peucedraminae) to all other members of the nine-primaried oscine clade.

Perhaps the most important component of any systematic study is defining a monophyletic group within which systematic relationships are to be addressed. If the monophyly of the group of interest is not certain, then a systematic study can be compromised (Lanyon 1994). The goal of this study was to test hypotheses of monophyly within the nine-primaried oscine assemblage using mtDNA sequences. We focus in particular on the five tribes that constitute the subfamily Emberizinae. To this end, we sampled representatives of each of the three outlined subfamilies, including several typically problematic taxa, and sequenced 1,929 base pairs of the mitochondrial genome. We analyzed these data with a series of phylogenetic reconstruction methods. From these analyses, we attempt to unravel the problematic relationships within the nine-primaried oscines and present a phylogenetic hypothesis as a framework for future systematic studies of the nine-primaried oscines.

METHODS

Taxa sampled.—We included samples of 35 species that traditionally are considered to be members of the nine-primaried oscine group, including 11 icterines, 7 emberizines, 3 parulines, 4 cardinalines, 9 thraupines, and 1 cardueline (species are listed in Appendix). We used a composite outgroup that included *Eremophila alpestris, Dicaeum trigonostigma, Peucedramus taeniatus, Lonchura bicolor,* and *Ploceus cucullatus.* All of these species are members of the superfamily Passeroidea (Sibley and Ahlquist 1990) and thus are believed to be reasonably close relatives

to the nine-primaried oscines. This sampling scheme encompasses much of the morphological diversity occurring within the nine-primaried oscines and close relatives and should provide a reasonable estimate of membership within and relationships among the major lineages.

DNA sequencing.—We extracted total genomic DNA from the specimens listed in the Appendix using either a Qiaquick tissue extraction kit (Qiagen) or by incubation in Chelex/Proteinase K, a modification of Ellegren's (1992) method. For cytochrome *b* (cyt *b*), we amplified DNA using the primers L14841 (Kocher et al. 1989) and H4a (Harshman 1996), and we sequenced an 894 base-pair (bp) segment using primers L14841, H15299 (Kocher et al. 1989); B3, B4, B5 (Lanyon 1994); and H4a. We amplified the NADH dehydrogenase subunit 2 (ND2) gene using the primers L5215 (Hackett 1996) and H6313 (Johnson and Sorenson 1998). We sequenced ND2 with these external primers and the internal primers L5758 (Johnson and Sorenson 1998) and H5776 (constructed for this study, 5'-TGGGARATGGAGGARAAGGC-3'). We conducted PCR in 50-μL reaction volumes using 0.5 μL Thermo flavus polymerase (Epicentre Technologies), 3 μL of 10 μM solution for each primer, 3.9 μL of 25 μM $MgCl_2$, 2.5 μL of 20× reaction buffer, 35 μL of distilled water, and between 1 to 5 μL of total genomic DNA extracts. A Perkin Elmer DNA Thermal Cycler 480 was used to perform the reactions, and the reaction conditions were one cycle of 3 min at 93°C, 1 min at 50°C, and 2 min at 72°C followed by 35 cycles of 1 min at 93°C, 1 min at 52°C, and 1 min and 20 s at 72°C. A 10-min extension at 72°C and a hold at 4°C followed these cycles. We prepared PCR products for sequencing using a Qiagen PCR Purification Kit and the manufacturer's protocols. We performed sequencing reactions using an ABI Prism Dye Terminator Reaction kit FS with manufacturer's protocols. We purified sequencing reactions using Centrisep columns packed with 0.05 g Sephadex (Sigma) in 0.8 mL water, following manufacturer's protocols. We dried sequencing products in a Centrivap vacuum concentrator and ran the products out on an acrylamide gel with an ABI 377 automated sequencing machine. We aligned resulting chromatograms of complementary strands and reconciled them using Sequencher 3.1 (GeneCodes). These sequences included the entire ND2 gene (1,035 bp) and a large portion (894 bp) of the cyt-*b* gene for each taxon.

Analysis: Gene comparisons.—We aligned sequences using Sequencher 3.1 (GeneCodes) and assessed variable and potentially phylogenetically informative sites using MEGA (Kumar et al. 1993). We compared these proportions between genes using a *z*-statistic approximation (Milton and Arnold 1990). Pairwise percentage sequence divergence as well as pairwise transition and transversion differences were calculated using MEGA (Kumar et al. 1993) for each codon position in each gene. To assess the likelihood of multiple substitutions of transitions and transversions at each codon position, we plotted the number of changes against percent sequence divergence for each type of change. To compare evolutionary rates between the two genes, we plotted the pairwise percent sequence divergence in ND2 against those for cyt *b*. We also reconstructed sequence evolution over the combined unweighted (all characters given equal weights) tree to estimate the transition/transversion ratio for both genes and to calculate the average number of changes per site for both genes. We compared the proportions of changes that were transitions using a *z*-statistic approximation (Milton and Arnold 1990). We were unable to estimate the "native" transition/transversion ratio at third positions for this data set using the method of Sturmbauer and Meyer (1992) because of the lack of pairwise comparisons between closely related species, but we include estimates from a separate study (Johnson and Lanyon 1999) of the grackles and allies (a clade within the nine-primaried oscines). We also estimated the transition/transversion ratios using maximum likelihood (see below).

Analysis: Weighting and phylogenetic methods.—To explore the sensitivity of tree topology to phylogenetic methodology and weighting scheme, we analyzed the sequence data using several different techniques. We used PAUP* (Swofford 1999) for all analyses. To determine if cyt *b* and ND2 contain a similar phylogenetic signal, we performed a partition-homogeneity test (Farris et al. 1994, Swofford 1999). For further comparison, we also analyzed each gene region separately for the parsimony and bootstrap analyses and compared topologies and level of resolution. Because the partition-homogeneity test indicated that differences in phylogenies derived from the two genes could be attributed to sampling error rather than true phylogenetic incongruence (Bull et al. 1993), we present major phylogenetic results from only analyses that included both gene regions combined.

In the parsimony analysis, we employed several weighting schemes to determine the sensitivity of the phylogenetic topology to differential weighting of transversions over transitions. These included equal weighting (1:1) and weighting of transversions over transitions by 2:1, 4.3:1, and 5:1 at third positions only (Yoder et al. 1996). We conducted a bootstrap analysis (Felsenstein 1985) for each weighting scheme using PAUP* with 1,000 fast replicates.

Using PAUP*, we evaluated likelihood values for various models over the tree obtained from the 5:1 transversion weighting of third positions. Using maximum likelihood, we estimated the transition/transversion ratio and the gamma shape parameter for rate heterogeneity using four rate categories. To choose a maximum-likelihood model for constructing a tree, first we evaluated whether a likelihood

model incorporating rate heterogeneity among sites was significantly better, using a likelihood-ratio test, than a model that did not incorporate rate heterogeneity. Next, we used likelihood-ratio tests to evaluate whether increasing parameters associated with substitution types could be statistically justified. Our goal was to find the simplest maximum-likelihood model that could not be rejected in favor of a more complex one. In this case, the gamma-corrected HKY85 model (Hasegawa et al. 1985), which incorporates rate heterogeneity (gamma shape parameter = 0.28) and two substitution types (transition/transversion ratio = 4.34), was chosen. We used the quartet puzzling option in PAUP* to search for the most likely tree under the above model. We also constructed neighbor-joining trees using Kimura two-parameter (Kimura 1980) and Log Det (Lockhart et al. 1994) genetic distances.

Because we found a large potential for multiple substitutions of transitions at third positions, we used a topology derived from transversion weighting of 5:1 at third positions as the best estimate of phylogenetic relationships among the nine-primaried oscines. To estimate the degree to which this topology was dependent on character composition, we conducted a full heuristic bootstrap search (Felsenstein 1985) with this weighting scheme. Likewise, we used a jackknife analysis (Lanyon 1985) to assess the degree to which this topology depends upon taxon composition.

To evaluate the robustness of traditional groupings (sparrows, warblers, blackbirds, tanagers, and cardinal-grosbeaks), we moved branches in MacClade (Maddison and Maddison 1992) to determine the number of additional steps that were required to make each group monophyletic. We also forced the mtDNA data to conform to the topologies of Raikow (1978), Bledsoe (1988), and Sibley and Ahlquist (1990) and determined the number of additional steps required. Using PAUP*, we tested likelihoods of alternative topologies statistically using the Kishino-Hasegawa (1989) likelihood-ratio tests with the model indicated above.

RESULTS

Molecular variation between genes.—Of 1,035 positions in ND2, 590 (56.8%) were variable and 508 (49.1%) were potentially phylogenetically informative. Of 894 base positions in cyt *b*, 393 (44.0%) were variable and 312 (34.9%) were potentially phylogenetically informative. Overall, cyt *b* displayed significantly less variation than did ND2 ($P < 0.0001$). This variation also translates into differences in the variation at the amino acid level: 47.2% for ND2 and 27.2% for cyt *b* ($P < 0.001$).

A clear pattern emerged in plots of accumulations of transitions and transversions at each position against overall percent divergence for each gene in pairwise comparisons (Fig. 1). At third positions, transitions accumulated up to approximately 15% sequence divergence, at which point accumulation ceased and the number of transitions dropped. In cyt *b*, accumulation of transitions appeared to level off below 10%. ND2 also showed higher levels of accumulation of transitions at first and second positions. However, transversions at third positions appeared to accumulate steadily in both cyt *b* and ND2 at approximately the same rate. Transversions at first positions also appeared to be higher in ND2 than in cyt *b*. In contrast, transversions at second positions appeared similar between ND2 and cyt *b*. These differences resulted in an overall faster rate of evolution in ND2 than in cyt *b* at the levels of divergence in this study (Fig. 2).

Reconstructed transition/transversion ratios over the unweighted combined tree (below) differed dramatically between genes. The reconstructed ratio for cyt *b* (1.6:1) was significantly lower than that for ND2 (2.9:1; $P < 0.0001$). The estimated transition/transversion ratios using a maximum-likelihood model with rate heterogeneity (shape parameters estimated from the data, four rate categories) were 2.46 for cyt *b* and 6.84 for ND2. This trend was opposite to the "native" ratios for third positions in the grackles and allies lineage using the method of Sturmbauer and Meyer (1992): 5:1 for cyt *b* and 4:1 for ND2 (Johnson and Lanyon 1999). This reversal of ratio magnitudes suggests that transitions in cyt *b* were more subject to multiple substitution than they were in ND2. Interestingly, the transition/transversion ratios in nine-primaried oscines were considerably lower than in some other groups of birds (e.g. 15:1 in dabbling ducks; Johnson and Sorenson 1998). The most common transitions for both genes were C to T, and A to G (Table 1). The reconstructed number of changes per site was 2.4 for cyt *b* and 3.6 for ND2, consistent with a higher rate of substitution for ND2.

Phylogenetic tree reconstruction.—In an unweighted (1:1) parsimony analysis of the two genes separately, consensus trees from ND2 showed slightly higher resolution than those for cyt *b*: 35 resolved nodes for ND2 and 31 for cyt *b*. In addition, the ND2 50% bootstrap to-

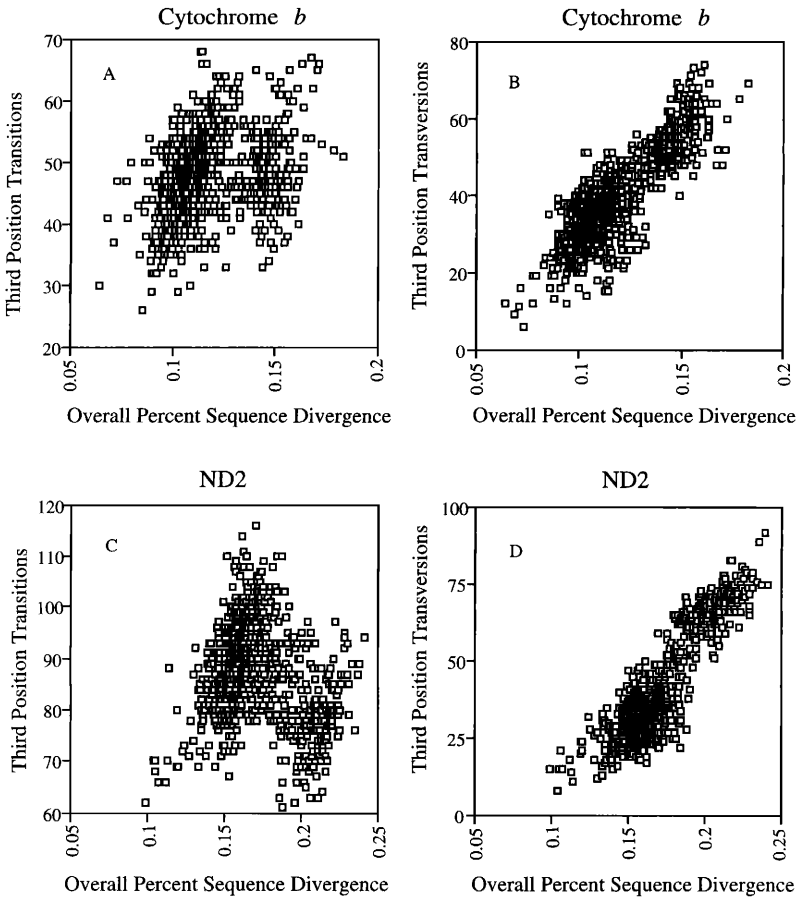

FIG. 1. Plots of transition and transversion substitutions in pairwise comparisons at each site against total percentage sequence divergence for cyt *b* and ND2. Similar plots for first and second positions for both genes were linear.

pology was more resolved than that for cyt *b*: 10 nodes supported above the 50% level for ND2 and 7 nodes supported above the 50% level for cyt *b*. None of the nodes that differed between the two trees was supported in the 50% bootstrap topology for either gene. Because the partition-homogeneity test (with no weighting) revealed that no significant incongruence occurred between phylogenies derived from the two genes ($P = 0.60$), we combined the two gene regions for the remaining analyses.

Combining gene regions in the analysis resulted in increased resolution and support. In an equally weighted (1:1) analysis, 35 nodes were resolved in the strict-consensus tree (Fig. 3A). More important, 13 nodes received support above the 50% bootstrap level, including two additional nodes not present in the analysis of the genes independently. In addition, all 13 nodes showed an increase in bootstrap support over the levels in the separate analyses.

Because the unweighted topology contained many nodes that were supported below the 50% bootstrap level, we wanted to determine the sensitivity of this topology to weighting scheme and method of analysis. In general, the results of the 1:1, 2:1, 4.3:1, and 5:1 (third positions only) analyses (Figs. 3A–D) showed remarkable similarity given the low levels of bootstrap support for many nodes. Nodes with high bootstrap support generally appeared in all trees irrespective of weighting scheme, whereas nodes with low bootstrap support did not. Of the 36 nodes in our maximum-likelihood tree (Fig. 3F), 22 (61.0%) occurred across all methods of analysis. Similarly, 21 of 35

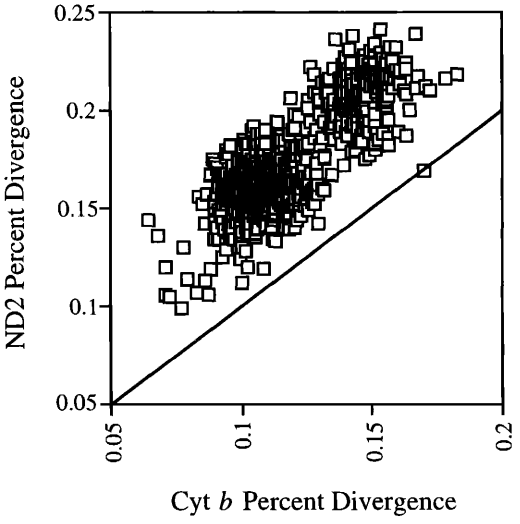

FIG. 2. Plot of percent sequence divergence in ND2 against that of cyt *b* in pairwise comparisons of all sequences. The line shows the expectation under the assumption that the two genes evolve at equal rates. Note that all points fall above the line, suggesting that at this level of divergence, ND2 accumulates substitutions at a higher rate.

TABLE 1. Base changes for each gene as reconstructed over the "Yoder" tree.

From	To A	C	G	T
		ND2		
A	—	262	740	146
C	331	—	60	1494
G	169	15	—	19
T	81	405	26	—
		Cytochrome *b*		
A	—	276	246	111
C	320	—	39	915
G	59	14	—	8
T	31	142	15	—

nodes (58.3%) in our distance tree (Fig. 3E) were stable across all parsimony analyses regardless of weighting scheme (see Fig. 4 for identification of stable nodes). Weighting third-position transversions 5:1 over other changes (Yoder et al. 1996; Fig. 3D) provided the topology with the greatest number of fully resolved nodes and generally higher levels of bootstrap support. The maximum-likelihood tree (Fig. 3F) shared 25 of the 34 ingroup nodes on this tree, and importantly, all of the major groupings of nine-primaried oscines and the relationships among them were consistent between these two trees (cf. Fig. 3D and 3F; groupings identified in Fig. 4). We use the tree derived from weighting third-position transversions 5:1 (hereafter the "Yoder" tree; Fig. 4) as our working phylogenetic hypothesis.

Phylogenetic relationships.—The monophyly of the nine-primaried oscines (Sibley and Ahlquist's [1990] Fringillidae, exclusive of *Peucedramus*) was strongly supported by bootstrap and jackknife pseudoreplicates. This node (node 1 in Fig. 4; Fringillinae + Emberizinae) was insensitive to weighting scheme or method of analysis and appeared in each tree that was generated (Fig. 3). The node uniting the more narrowly defined New World nine-primaried oscines (node 2 in Fig. 4; Sibley and Ahlquist's [1990] Emberizinae), with the exception of one taxon, also was well supported by bootstrapping and jackknifing, appearing across all analyses. Surprisingly, *Euphonia* (traditionally considered a thraupine) grouped with *Carduelis* in all analyses, and these two were sister to all other nine-primaried oscines. This relationship was highly supported by jackknife and bootstrap pseudoreplicates. Constraining the Emberizinae assemblage (node 2 in Fig. 4) to include *Euphonia* in a basal position yielded a significantly worse tree (Kishino-Hasegawa test, $P = 0.0247$), requiring 28 additional steps.

Within the subfamily Emberizinae, the traditional nine-primaried oscine groupings (tribes) in general were retained, but none appear to be strictly monophyletic. In the Yoder tree, and also in the maximum-likelihood and 2:1 and 4.3:1 parsimony analyses (Figs. 3B, C, D, F), *Calcarius* (a putative emberizine) occurred as sister to (outside) the more narrowly defined nine-primaried oscine clade. Placement of *Calcarius* shifted rather dramatically across weighting schemes and analytical method, however, grouping with *Sturnella* near the base of the nine-primaried clade under equal weighting (Fig. 3A) and with the Cardinalini when using distance methods (Fig. 3E). Although placement of *Calcarius* is ambiguous, it does not likely belong within the Emberizini. Constraining the sparrow assemblage to include *Calcarius* requires an additional 16 steps and results in a significantly worse topology (Kishino-Hasegawa test, $P = 0.0013$).

Our data suggest that the tribe Cardinalini,

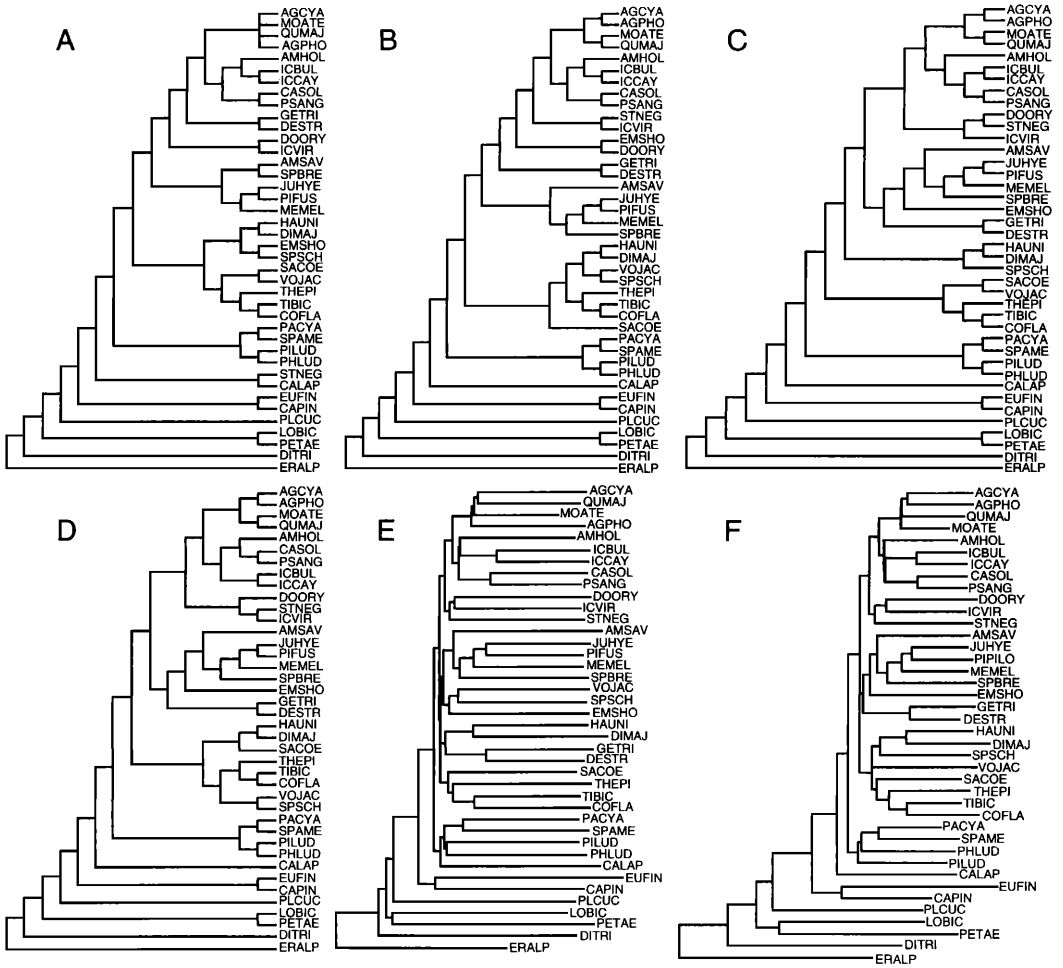

FIG. 3. Comparisons of phylogenies derived from alternative weighting schemes and methods of analysis: (A) Equally weighted parsimony (1:1), strict consensus of two trees, l = 5,913, RC (rescaled consistency index) = 0.071. (B) 2:1 transversion-weighted parsimony, one tree, l = 7,608, RC = 0.084. (C) 4.3:1 transversion-weighted parsimony, one tree, l = 11,407, RC = 0.171. (D) parsimony with 5:1 transversion weighting at third positions only, one tree, l = 11,578, RC = 0.091. (E) neighbor-joining tree with Kimura two-parameter distances. (F) maximum-likelihood tree with HKY85 model of substitution, estimated base and transversion frequencies, and no rate heterogeneity. See Appendix for species identification codes.

as currently recognized, is both paraphyletic and polyphyletic. Three cardinalines (*Pheucticus, Passerina,* and *Spiza*) plus the tanager *Piranga* formed a group that was sister to all other members of the New World nine-primaried oscine clade. This group, evident across all analyses, received low bootstrap support but relatively high jackknife support (Fig. 4). Forcing *Piranga* into a more traditional placement, sister to the tanager clade, required 24 additional steps but did not result in a significantly poorer topology (Kishino-Hasegawa test, $P = 0.0794$; although nonparametric Templeton [$P = 0.0016$] and winning-sites [$P = 0.0025$] tests were significant). The placement of a fourth putative cardinaline, *Saltator,* was less stable but fell consistently within Thraupini.

Despite the fact that the constituents represent multiple tribes of oscines, a thraupine group (exclusive of *Piranga* and *Euphonia*) appeared in many of the analyses, being present as a clade in the maximum-likelihood and most of the parsimony analyses. In the Yoder tree, the Thraupini forms a poorly supported clade

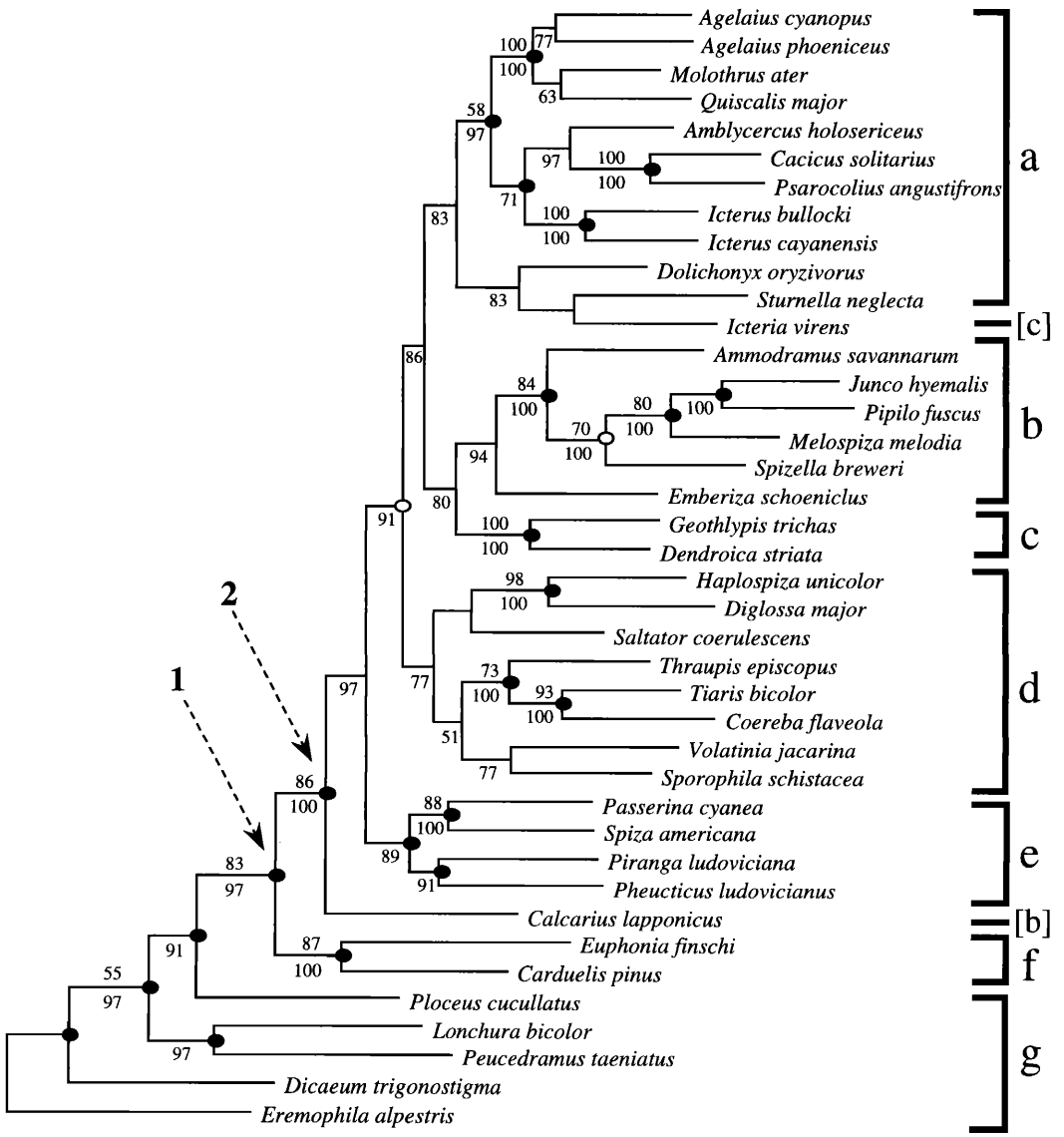

FIG. 4. Single most-parsimonious tree (l = 11,578) resulting from unweighted parsimony of first and second positions and 5:1 transversion weighting of third positions only ("Yoder" tree; Yoder et al. 1996). Branch lengths are proportional to the number of reconstructed changes. Bootstrap values from 100 full heuristic search replicates are indicated above branches that received more than 50% support; values obtained from jackknifed pseudoreplicates are shown below. Filled ovals indicate nodes that are stable across all trees (Fig. 3). An additional two nodes, found in all but the equally weighted parsimony tree (Fig. 3A), are depicted by open ovals. Nodes 1 and 2 represent the family Fringillidae (excepting *Peucedramus*) and the subfamily Emberizinae, respectively (sensu Sibley and Monroe 1990). Labeled clades are identified as follows: a, tribe Icterini; b, tribe Emberizini; c, tribe Parulini; d, tribe Thraupini; e, tribe Cardinalini; f, subfamily Fringillinae; and g, outgroups. Taxonomic placement of *Icteria virens* (c) and *Calcarius lapponicus* (b) is unresolved.

that is sister to a sparrow-warbler-blackbird clade. Two pairs of genera within Thraupini grouped strongly in bootstrap and jackknife analyses, across weighting schemes, and across methods of analysis: *Tiaris* with *Coereba* and *Haplospiza* with *Diglossa*. The "tanager-finches" (represented by *Tiaris, Volatinia, Sporophila,* and *Haplospiza*) are a consistent and stable compo-

nent of the thraupine clade across all analyses. Moving them to their traditional placement within the emberizines required 78 additional steps (Kishino-Hasegawa test, $P < 0.0001$). Likewise, reconstructing more traditional thraupine and cardinaline arrangements by moving *Saltator* to the cardinal-grosbeaks and forcing *Euphonia* and *Piranga* into the tanagers required 63 additional steps and resulted in a significantly worse topology (Kishino-Hasegawa test, $P < 0.0001$). Forcing only *Euphonia* into a basal position in the thraupine assemblage yielded a tree that was 59 steps longer (Kishino-Hasegawa test, $P < 0.0001$).

A sparrow-warbler-blackbird clade appeared in the Yoder tree and in all other parsimony analyses (Figs. 3A–D) but was not evident in neighbor-joining or maximum-likelihood analyses. This node was poorly supported by bootstrapping but well supported by jackknife replicates. In the Yoder and 4.3:1 trees, Parulini and Emberizini were sisters, but in general, relationships among these three tribes remain unclear. The New World sparrows (exclusive of *Calcarius* and *Emberiza*) also formed a monophyletic group in all trees receiving high bootstrap and jackknife support in the Yoder tree. The position of *Emberiza* was unstable to weighting and method of analysis. In the Yoder, 4.3:1, and maximum-likelihood trees, it has a traditional placement, sister to all other sparrows. However, in the equally weighted and neighbor-joining trees it joined Thraupini, and in our 2:1 weighted analysis it fell within Icterini. The two wood-warblers (exclusive of *Icteria,* traditionally considered an aberrant paruline) formed a monophyletic group that was well supported and appeared across all methods of analysis. The monophyly of blackbirds (exclusive of *Dolichonyx* and *Sturnella*) appeared in all trees irrespective of weighting scheme or method of analysis. *Sturnella, Dolichonyx* (both putative icterines), and *Icteria* tended to group together, forming a clade in the Yoder tree, 4.3:1 tree, neighbor-joining analysis, and maximum-likelihood analysis. *Icteria* grouped with either *Dolichonyx* (1:1 weighting) or *Sturnella* (2:1 weighting) in the other two trees. Forcing *Icteria* into the Parulini results in a tree in which both the Icterini and the Parulini are monophyletic. This tree was only seven steps longer and not significantly worse (Kishino-Hasegawa test, $P = 0.0623$) than the Yoder tree.

To summarize, the Yoder topology recovered the six major groups identified in most traditional classifications: blackbirds and allies, New World sparrows, wood-warblers, tanagers and tanager-finches, cardinal-grosbeaks, and the true finches; however, this tree indicated that few of these groups are monophyletic as presently understood. In particular, placements of *Piranga, Saltator, Icteria, Calcarius,* and *Euphonia* are at odds with any previous traditional (sequential) taxonomic arrangements. We explored forcing our data to conform to the topologies of previous among-group arrangements (Fig. 5). We pruned the Yoder tree, removing two problematic taxa (*Icteria virens* and *Calcarius lapponicus*) and the outgroups. Constraining to Bledsoe's (1988) topology required only 7 additional steps (Kishino-Hasegawa test, $P = 0.7488$), whereas forcing the topology of Sibley and Ahlquist (1990) required 11 additional steps (Kishino-Hasegawa test, $P = 0.0064$). Raikow's (1978) topology, depending on how the polytomy was resolved, required either 6 (Kishino-Hasegawa test, $P = 0.0823$) or 13 (Kishino-Hasegawa test, $P = 0.0484$) additional steps (see Table 2).

Discussion

Using mtDNA sequences, we resolved phylogenetic relationships among and within many of the major lineages of nine-primaried oscines. This phylogeny was not strongly supported by bootstrap replicates, however, despite being based on approximately 2,000 bp of mtDNA sequence and including representatives of most previously identified nine-primaried oscine lineages. We suggest that one major contributor to this lack of support was the high level of homoplasy that we observed in the data. The bootstrap technique (Felsenstein 1985) is subject to estimation bias with high levels of homoplasy, such that the bootstrap value seriously underestimates the confidence level when homoplasy is high (Zharkikh and Li 1992, 1995). In our study, we intentionally chose widely divergent taxa so that we covered a wide range of nine-primaried oscine lineages. Because these taxa are divergent, the number of undetected (and detected) multiple substitutions is high, and this will reduce the

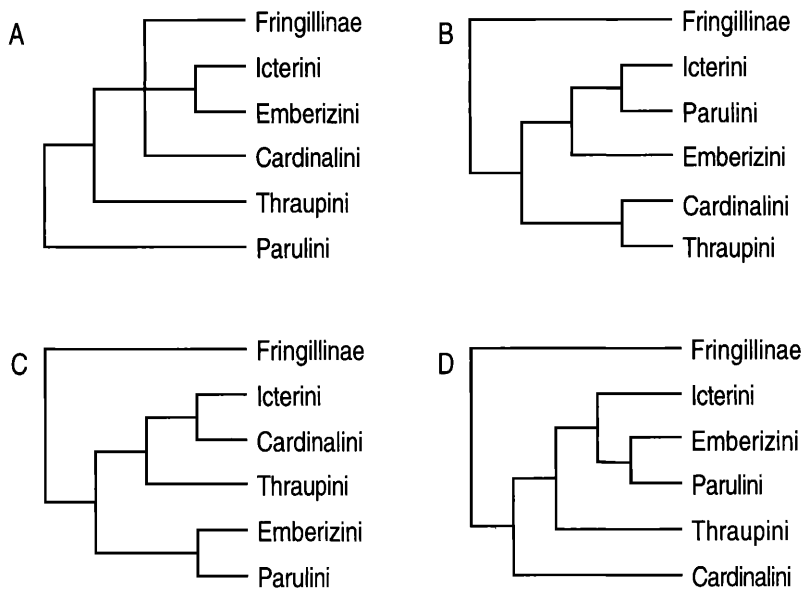

FIG. 5. Competing recent phylogenetic hypotheses of fringillid relationships. (A) After Raikow (1978), although described as cladistic, relationships are not entirely based on character state data (Bledsoe and Raikow 1990); (B) after Bledsoe's (1988) unjackknifed FITCH tree; (C) after Sibley and Ahlquist's (1990: figure 352) FITCH tree; (D) this study.

bootstrap estimates of confidence level. In such cases, we alternatively examine the stability of tree topology to weighting scheme, method of analysis, and to jackknife pseudoreplicates. In this study, many groupings were very stable to weighting scheme and method of analysis even though they did not appear in more than 50% of bootstrap pseudoreplicates. We suggest that the low level of bootstrap support observed is not necessarily an indicator of lack of phylogenetic signal in the data.

Because we sequenced DNA from only 40 of some 1,000 possible species, the analysis clearly suffered from problems related to inadequate sampling of taxa (Lecointre et al. 1993, Graybeal 1998, Hillis 1998). Much of the lack of strong phylogenetic resolution was due to the fact that the tree included many long branches connected by short internodes. It is well appreciated that one way to increase resolution and support is to sample additional taxa to break up long branches (Felsenstein 1978; Hillis 1996, 1998; Graybeal 1998). Clearly, many taxa should be added to this analysis both to further refine membership of major groups and to increase support for relationships among the major groups. The "low stemminess" depicted in our neighbor-joining tree (Fig. 3E) was an artifact of taxon sampling but also could indicate that the diversification of an ancestral species into the major nine-primaried oscine lineages occurred at approximately the same time during a rapid radiation event. The addition of more taxa to those included in this study should distinguish between these alternative explanations.

Although some authors have questioned the relative utility of cyt *b* for phylogenetic studies compared with other mitochondrial protein-coding genes (Meyer 1994), others have found that cyt *b* and ND2 provide extremely similar resolution and support (Johnson and Sorenson 1998). In waterfowl, cyt *b* and ND2 appear to be evolving at similar rates and with similar types of substitutions; however, in passerines, ND2 appears to accumulate detected substitutions more rapidly at higher divergences than does cyt *b* (Hackett 1996, Johnson and Lanyon 1999). This can result in slightly more resolution and support for trees derived from ND2 versus cyt *b*. In this study, we found that ND2 performs slightly better than cyt *b* in terms of providing support for various nodes. However, levels of overall homoplasy between the two genes were similar (rescaled consistency index: cyt *b* = 0.081, ND2 = 0.069), and the

TABLE 2. Kishino-Hasegawa tests of competing tree topologies. Clade designations refer to Figure 4. Constraints 9 to 13 are based on a partial data set in which two problematic taxa (*Icteria* and *Calcarius*) were removed along with the outgroups. Values were obtained using the HKY85 model (Hasegawa et al. 1985) with the transition/transversion ratios and the gamma shape parameters estimated from the data.

Constraint	Length	$-\ln L$	P
(1) Yoder tree	5,926	25,782.18	Best
(2) Move *Icteria* to Parulini (clades a and c monophyletic)	5,933	25,797.65	0.0623
(3) Move *Calcarius* to Emberizini	5,942	25,819.44	0.0013
(4) Move Tanager-finches to Emberizini (*Haplospiza, Tiaris, Sporophila, Volatinia* to clade b)	6,004	25,921.03	<0.0001
(5) Move Tanager-finches and *Calcarius* to Emberizini (clade b monophyletic)	6,018	25,947.70	<0.0001
(6) Move *Saltator* to Cardinalini, *Piranga* and *Euphonia* to Thraupini (clade e monophyletic)	5,989	25,864.67	<0.0001
(7) Move *Euphonia* to Emberizinae (node 2)	5,954	25,798.27	0.0247
(8) Move all to reflect AOU (1998) families	6,102	26,045.70	<0.0001
(9) Yoder tree, pruned	4,540	20,560.00	Best
(10) Bledsoe (1988) tree	4,547	20,561.47	0.7488
(11) Sibley and Ahlquist (1990) tree	4,551	20,593.80	0.0064
(12) Raikow (1978) tree (Fringillinae and Cardinalini sisters)	4,546	20,577.13	0.0823
(13) Raikow (1978) tree (all alternative polytomy rearrangements)	4,553	20,582.64	0.0484

increased support was by no means dramatic. By combining genes, a more highly supported and resolved topology was obtained, suggesting that each gene provided a similar phylogenetic signal and that combining genes strengthened this signal.

Phylogenetic implications.—The monophyly of the nine-primaried oscine assemblage (excepting *Peucedramus*) is strongly supported; however, this result should be interpreted with caution (Sanderson 1996). Although nine-primaried oscines are loosely defined by a suite of morphological and physiological characters (see Raikow 1978), none of the characters can be considered strict synapomorphies for the group. For example, several lineages typically excluded from the nine-primaried oscines do have nine functional primaries per wing (e.g. larks and wagtails). In our arrangement, *Peucedramus* (also a nine-primaried bird) is not sister to all other members of the group, as Sibley and Ahlquist (1990) had speculated, but instead turns up as sister to an estrildid finch (*Lonchura*). Clearly, more work is needed in this area.

We also cannot conclude that the tribes identified represent all nine-primaried oscine radiations. *Calcarius* has always been considered a New World sparrow, yet it can be distinguished (along with *Plectrophenax*) from that group on osteological grounds (Tordoff 1954b). An allozyme study (Avise et al. 1980) appears to confirm that *Calcarius* is genetically distant from other North American sparrows. In none of our analyses was *Calcarius* placed among the emberizines; instead, it repeatedly occurred near the base of the tree. In the Yoder tree (Fig. 4), *Calcarius* is sister to a clade that encompasses all of the tribes within Emberizinae. That *Calcarius* species look like sparrows but are not in the sparrow lineage suggests that they represent a previously unrecognized early radiation event and convergence upon sparrow morphology. A cyt-*b* analysis that includes all members of *Calcarius* is consistent with this notion (J. Klicka unpubl. data).

Owing to the paucity of robust morphological characters, few hypotheses concerning relationships among tribes in the nine-primaried oscines have been proposed. Of those shown, the three based on molecular characters (Figs. 5B–D) place Fringillinae (fringillines, carduelines, drepanidinines) at the base of the nine-primaried oscine tree. Raikow's (1978) tree varies (in part) because it was rooted, on the basis of "primitive appendicular musculature," with Parulini. Our tree (part d in Fig. 4) is most similar, but not significantly better, than that of Bledsoe (1988; our Fig. 5B) in that within Emberizinae, sparrows, warblers, and

blackbirds form a clade exclusive of tanagers and cardinal-grosbeaks. However, warblers and blackbirds are sisters in Bledsoe's tree, and warblers and sparrows are sisters in ours; this is the only node that our tree and Sibley and Ahlquist's (1990; our Fig. 5C) have in common. Tordoff also (1954a) suggested that both jaw musculature and bony palate structure supported a paruline-emberizine relationship. Sibley and Ahlquist (1990) were able to derive few conclusions from their data concerning relationships among the tribes of the subfamily Emberizinae. The one sister relationship they resolved was that of Cardinalini-Icterini, a pairing that we find unlikely (Table 2).

A strict interpretation of our working phylogenetic hypothesis (Yoder tree; our Fig. 4) indicates that not one tribe among the five lineages comprising the Emberizinae is monophyletic. Our results confirm what earlier molecular studies (Bledsoe 1988, Sibley and Ahlquist 1990) have suggested: that considerable room for incongruence between molecular and morphological phylogenies exists and that molecular data may improve upon taxonomies that are based solely on morphological and anatomical assessments. Upon closer inspection, however, it is apparent that the major historical groupings hold up remarkably well, even though several genera are misplaced. Despite placement of *Sturnella* and *Dolichonyx* outside of the group in this study, the tribe *Icterini* may yet prove to be monophyletic. Indeed, Sclater's (1886) Icteridae contains the same taxa currently classified as Icterini, and all of Ridgway's (1902) icterid genera still are considered to be members of the group. Likewise, Sharpe's (1885) boundaries for wood-warblers (Mniotiltidae) have remained mostly intact, changed only by the recent addition of the Wrenthrush (*Zeledonia coronata*), formerly of the monotypic family Zeledoniidae (Sibley 1968, AOU 1983). Our small sample size allows us to conclude little regarding New World warblers. *Icteria* was placed unequivocally within Parulini by DNA-DNA hybridization comparisons (Sibley and Ahlquist 1982), suggesting that additional sampling of more varied warbler taxa (e.g. *Seiurus* and *Myioborus*) could alter our results. The position of *Icteria* in our tree may reflect an affinity between blackbirds and warblers (sensu Bledsoe 1988; our Fig. 5B) that our limited data were unable to detect.

Except for the previously discussed *Calcarius*, the emberizines (Sibley and Monroe 1990) are well defined. The precise position of *Emberiza* remains unclear. Based on allozyme differences, Watada et al. (1995) concluded that the large genus *Emberiza* represents an early Old World radiation. Our tree, with *Emberiza* embedded deep within the nine-primaried oscine clade, is consistent with the traditional interpretation in which *Emberiza* is of New World origin with a secondary radiation into the Old World. That sparrows (narrowly defined) are in a clade apart from tanagers and cardinal-grosbeaks is consistent with the interpretation that placement of sparrows with tanagers and cardinal-grosbeaks in most sequential classifications reflects convergence in bill morphology and related characters rather than a shared evolutionary history.

The tribes Cardinalini and Thraupini are less well defined by our data, as they have been in most other classifications. The Cardinalini appears to represent a real assemblage, although some memberships are unresolved. On the basis of jaw musculature, the monotypic *Spiza* was thought to be a member of the Icterini (Beecher 1953); based on palatal structure (Tordoff 1954b) and serology (Stallcup 1954), however, *Spiza* was determined to be a cardinaline, a view consistent with our results. The genus *Saltator* historically has been entrenched within the Cardinalini (e.g. Ridgway 1901) but may in fact be a thraupine. Although its exact position within the Thraupini is unstable, it is placed within this group in all of our analyses. Sushkin (1924) considered *Saltator* a "thick-billed tanager," and a few others (Mayr and Amadon 1951, Tordoff 1954b) thought it provided a "transition" between Thraupini and Cardinalini. Although affinities with another tribe had been suggested for *Saltator*, such was not the case with the genus *Piranga*. Long considered to be a "classic" tanager (Ridgely and Tudor 1989), every analysis in our study places this genus as sister to the cardinaline *Pheucticus*. Preliminary cyt-*b* analysis of an expanded cardinaline-thraupine data set (J. Klicka and K. Burns unpubl. data) also supports the placement of *Piranga* with the cardinal-grosbeaks. The tanager topology of Burns (1997) suggests that the associated genera *Habia* and *Chlorothraupis* also have affinities with the Cardinalini. If *Piranga* is indeed a cardinaline, then the tribe

Thraupini will lose its only Nearctic representation and become an exclusively Neotropical assemblage.

Another "tanager problem" concerns the genera *Euphonia* and *Chlorophonia*. Although long considered to be atypical among tanager forms (e.g. Ridgway 1902) these taxa have, because of their bright plumage coloration and frugivorous diet, traditionally been assigned to that group. In a recent and thorough molecular study of tanager relationships, Burns (1997) excluded these genera from this assemblage. Our study provides strong support for this finding, additionally suggesting that *Euphonia* (and by association *Chlorophonia*) represents either a derived cardueline form or a basal, previously unrecognized radiation within the nine-primaried oscine clade. Similarities between euphonias and cardueline finches have been noted previously. In comparing *Euphonia affinis* with *Spinus psaltria*, Dickey and van Rossem (1938: 546) remarked "That such a close parallel in size and color exists between two members of separate families is remarkable enough, but when the resemblance extends still further to call-notes, general habits, and even to the occurrence of the annual molt in summer instead of fall, it seems extraordinary." Because of the taxonomic emphasis placed on feeding specializations and "trophic zones" (Bledsoe 1988), most earlier workers overlooked the less obvious alternative that *Euphonia* may represent "goldfinches" that secondarily have become fruit specialists.

It seems that "tanagers" of one form or another have been central to many of the problems in nine-primaried oscine taxonomy over the course of the last century. As a case in point, the eight (putative) thraupine taxa in our phylogeny represent no less than four different songbird families (Emberizidae, Coerebidae, Cardinalidae, and Thraupidae) according to the most recent AOU check-list (1998). The DNA-DNA hybridization studies of Bledsoe (1988), Sibley and Ahlquist (1985, 1990), and now our work based on mtDNA sequences, suggest that incongruence between molecular studies and traditional taxonomy are most frequently the result of convergence upon particular feeding morphologies and dramatic within-group divergence (see also Sibley 1970:108). Thraupines, for example, historically have been considered to be frugivores primarily (Sibley 1970); however, it is increasingly clear that the tribe occupies several of the trophic niches typical of other nine-primaried oscines (Bledsoe 1988), including an assemblage of sparrow-like birds, the "tanager-finches" (Sibley and Ahlquist 1985, Bledsoe 1988), and a group of nectarivores. Even within the tribe, these feeding types have evolved multiple times (Burns 1997, this study). The great evolutionary plasticity of bill form and function is well known from the radiations of Hawaiian honeycreepers and Galapagos finches. As the true members of the thraupine assemblage continue to be identified, it is becoming clear that tanagers represent such a radiation on a continental scale, the "island" of South America.

We consider the phylogeny presented here to be a working hypothesis that can be used to guide further study of the nine-primaried oscines. Because the first (and perhaps most critical) step in any phylogenetic analysis is to identify the limits for the group of study, we hope that this work will aid in delimiting major groups and provide focus for future studies. For example, it appears clear that the tanagers (as traditionally defined; see Burns 1997) are not a monophyletic group. Future studies involving this lineage should proceed cautiously when inferring relationships among putative members. Other lineages, such as the New World sparrows, blackbirds, and wood-warblers (all as narrowly defined), appear to be upheld. That they also appear to share a common ancestor suggests that they would make appropriate outgroups for one another in future studies. This work again demonstrates the limitations of using morphological characters to define relationships within this assemblage and makes clear how much more work of this type is required if we are to truly understand the evolutionary history of New World oscines.

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APPENDIX. Species names, voucher numbers, and localities of origin for taxa sequenced.

Species	Code	Museum[a]	Catalog field no.	Locality	Collection date
Eremophila alpestris	ERALP	BMNH	JK9480	USA, Montana, Hill Co.	20 June 1994
Dicaeum trigonostigma	DITRI	FMNH	358515	Philippines, Sibuyan	1 November 1992
Peucedramus taeniatus	PETAE	FMNH	MEX396	Mexico (voucher at UNAM)	30 April 1989
Lonchura bicolor	LOBIC	FMNH	357366	Congo, Haut-Zaire, Ituri	16 June 1991
Ploceus cucullatus	PLCUC	FMNH	357374	Congo, Haut-Zaire, Ituri	16 June 1991
Carduelis pinus	CAPIN	BMNH	X7293	USA, Minnesota, St. Louis, Co.	21 January 1995
Euphonia finschi	EUFIN	FMNH	389276	Brazil, Roraima	12 February 1992
Calcarius lapponicus	CALAP	BMNH	JDW0062	USA, Minnesota, Wilkin Co.	27 April 1996
Pheucticus ludovicianus	PHLUD	BMNH	X7253	USA, Minnesota, Brown Co.	9 May 1994
Piranga ludoviciana	PILUD	BMNH	JK94105	USA, Montana, Missoula Co.	25 June 1994
Spiza americana	SPAME	BMNH	JK95047	USA, Texas, Cameron Co.	16 July 1988
Passerina cyanea	PACYA	BMNH	JK94162	USA, Minnesota, Isanti Co.	5 August 1992
Sporophila schistacea	SPSCH	LSUMZ	B-22584	Bolivia, La Paz Dept., Prov. B. Saavedra	12 July 1993
Volatinia jacarina	VOJAC	FMNH	ANK228	Bolivia (voucher in Santa Cruz)	November 1988
Coereba flaveola	COFLA	BMNH	JK95006	Bahamas, Long Island	September 1994
Tiaris bicolor	TIBIC	BMNH	JK95001	Bahamas, Long Island	September 1994
Thraupis episcopus	THEPI	FMNH	339708	Venezuela, Falcon	5 November 1988
Saltator coerulescens	SACOE	FMNH	ANK170	Bolivia (voucher in Santa Cruz)	November 1988
Diglossa major	DIMAJ	FMNH	339722	Venezuela, Bolivar	22 November 1988
Haplospiza unicolor	HAUNI	FMNH	5186	Brazil, Sao Paulo	December 1991
Emberiza schoeniclus	EMSCH	UWBM	SAR6242	Kamchatka (Kamchatskaya, Oblast)	24 July 1992
Spizella breweri	SPBRE	BMNH	JK9465	USA, Montana, Chouteau Co.	19 June 1994
Melospiza melodia	MEMEL	FMNH	341624	USA, Illinois, Cook Co.	28 March 1987
Pipilo fuscus	PIFUS	BMNH	RMZ2373	USA, Arizona, Cochise Co.	20 June 1988
Junco hyemalis	JUHYE	LSUMZ	B14356	USA, California, San Bernardino Co.	9 December 1989
Ammodramus savannarum	AMSAV	BMNH	JK9456	USA, Montana, Chouteau Co.	19 June 1994
Icteria virens	ICVIR	BMNH	JK95141	USA, Texas, San Patricio Co.	23 September 1973
Sturnella neglecta	STNEG	FMNH	330039	USA, California, Riverside Co.	20 May 1986
Dolichonyx oryzivorus	DOORY	FMNH	334721	Bolivia, Santa Cruz, Chiquitos	5 November 1987
Icterus cayanensis	ICCAY	FMNH	334609	Bolivia, Santa Cruz, Chiquitos	10 November 1987
Icterus bullocki	ICBUL	FMNH	341938	USA, California, Monterey Co.	31 May 1988
Psarocolius angustifrons	PSANG	FMNH	324068	Peru, Madre de Dios	5 November 1985
Cacicus solitarius	CASOL	FMNH	324091	Peru, Madre de Dios	12 November 1985
Amblycercus holosericeus	AMHOL	LSUMZ	98900	Peru, Puno	12 October 1980
Quiscalus major	QUMAJ	FMNH	341918	USA, Louisiana, Cameron Pa.	28 April 1988
Molothrus ater	MOATE	FMNH	350707	USA, Illinois, Cook Co.	25 August 1989
Agelaius phoeniceus	AGPHO	FMNH	341893	USA, Louisiana, Cameron Pa.	28 April 1988
Agelaius cyanopus	AGCYA	FMNH	334636	Bolivia, El Beni, Laguna Suarez	19 November 1987
Geothlypis trichas	GETRI	BMNH	JK92119	USA, Oregon, Douglas Co.	30 May 1992
Dendroica striata	DESTR	BMNH	X7246	USA, Minnesota, Hennepin Co.	11 September 1994

[a] BMNH = James Ford Bell Museum of Natural History, FMNH = Field Museum of Natural History, LSUMZ = Louisiana State University Museum of Zoology, UWBM = University of Washington Burke Museum, UNAM = Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autónoma de México