Rhodinocichla rosea is an Emberizid (Aves; Passeriformes) Based on Mitochondrial DNA Analyses

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The systematic position of the avian species Rhodinocichla rosea is unclear. Recent opinions are that it is either a mockingbird (family Mimidae) or a tanager (Thraupinae; Emberizidae). In either case, it would be an atypical member of the family. We sequenced approximately 600 bases of the mitochondrial cytochrome oxidase I (COI) gene of Rhodinocichla, several mimids, tanagers, and other passerines. We used maximum likelihood (ML), distance and parsimony approaches to analyze the sequences and concluded that Rhodinocichla belongs to the family Emberizidae. Phenotypic characteristics that suggested its relationship with mimids are the product of convergent evolution. The precise relationships of Rhodinocichla within the Emberizidae could not be resolved. Short internal branches in ML and distance trees suggested, as did earlier genetic studies, that the radiation of that family was explosive. Apparently, the extent of the tanagers as a higher taxon needs to be clarified. Our analysis of the evolutionary dynamics of avian COI suggested that its usefulness for phylogenetic studies is limited because silent positions saturate rapidly and replacement substitutions are rare. Thus, our data indicate that COI nucleotide data will be most useful in intraspecific investigations, while other data suggested its usefulness at the interordinal level.

INTRODUCTION

Cases of convergent evolution demonstrate how alternative evolutionary pathways can lead to a single phenotypic solution and help identify constraints limiting the evolution of phenotypes. But convergent evolution, because it produces similarities between organisms not resulting from common ancestry, can mask the genealogical relationships of taxa. In birds, there are several well-documented examples of convergence in morphology, behavior, and other phenotypic traits (e.g., Gill, 1990). However, in many other cases, the phylogenetic relationships of taxa are unclear, and the convergent nature of specific phenotypic attributes is uncertain. This is the case with the rosy thrush-tanager, Rhodinocichla rosea.

The rosy thrush-tanager is the only member of the highly distinctive genus Rhodinocichla. It is a medium-sized neotropical passerine (total length, 19–20 cm; weight, 43–52 g) with several geographically localized and widely separated populations (Isler and Isler, 1987). The species was described by Lesson (1832) as a Furnariidae, based on superficial similarities with the then-known members of this family of primitive songbirds. Soon after, it was recognized as belonging to the oscines (Hartlaub, 1853), a large group of derived passerines (see Fig. 1). But Rhodinocichla also proved to possess a mosaic of phenotypic characters that made it difficult to assign the genus to a particular family. Clark (1913), Skutch (1962), Eisenmann (1962), Clark (1974), and Raikow (1978) presented and discussed morphological, anatomical, myological, and behavioral peculiarities of the species. Over the last 100 years, the thrush-tanager has been classified with the wrens (Troglodytidae), the wood-warblers (Parulinae; Emberizidae), and, most frequently, the mockingbirds (Mimidae) or the tanagers (Thraupinae; Emberizidae) (e.g., Sharpe, 1881; Ridgway, 1902; Hellmayr, 1936; Eisenmann, 1962; Skutch, 1962; A.O.U., 1983; Webster, 1988). These various families and subfamilies are not closely related in either traditional classifications (e.g., Wetmore, 1960; A.O.U., 1983) or that presented by Sibley and Ahlquist (1990; Fig. 1). For instance, if one accepts Sibley and Ahlquist’s (1990) estimates and calibration of single copy nuclear DNA divergence, the mimids and the tanagers last shared a common ancestor 20–30 MY ago. This date for a mimid-tanager split conforms to fossil evidence suggesting that most oscine families were probably established well before the end of the Miocene (Brodkorb, 1971; Feduccia, 1980; Olson, 1985).

The essential problem with previous discussions of the taxonomic affinities of Rhodinocichla was that the
Rhodinocichla IS AN EMBERIZID

Suboscines (incl. Furnariidae)

Corvida (incl. Vireonidae)

Mimidae

Turdinae

Troglodytidae

Parulinae

Cardinalinae

Thraupinae

New World nine-primaried oscines (Emberizidae)

Oscines


characters considered were often not analyzed in an appropriate context. First, the homologous nature of several shared traits used to infer relationships had not been ascertained. Second, character similarities between taxa were typically taken as evidence of relationships, even though no consideration was given to whether the traits were ancestral or derived. A definitive taxonomic assignment of Rhodinocichla, independent of phenotypic characters, should help us to understand the evolution of its numerous anatomical and behavioral peculiarities.

Mitochondrial DNA (mtDNA) sequence data have been fruitfully utilized to resolve avian taxonomic enigmas (Edwards et al., 1991; Avise et al., 1994a; Zimmer et al., 1994; but see Avise et al., 1994b). We investigated the phylogenetic relationships of Rhodinocichla by analyzing roughly 600 base pairs (bp) of the gene coding for the first subunit of the cytochrome c oxidase protein (COI or Cox1). The analyses strongly indicate that Rhodinocichla is a tanager, not a mimid. The data also allowed us to investigate the evolutionary dynamics of avian COI and its general utility for phylogenetic studies of birds.

MATERIALS AND METHODS

Samples. Our samples (Table 1, see also Fig. 1) included Rhodinocichla rosea, one species each representing Troglodytidae, Turdinae, Parulinae, and Cardinalinae, and three species each of Mimidae and Thraupinae. For most species, we sequenced two individuals, and in all but one case we surveyed two or more members of a subfamily (range 2–5). Further, we included two species of Vireonidae as outgroups. Until recently, Vireonidae were thought to be nine-primaried oscines (e.g., A.O.U., 1983), but they are now known to be part of the corvine radiation (Johnson et al., 1988; Sibley and Ahlquist, 1990). Thus, vireos were an appropriate outgroup for our analyses (Fig. 1). Most tissue samples were collected as biopsies from live birds and preserved in a salt-DMSO solution (Seutin et al., 1991, 1994); other samples came from sacrificed individuals (see Appendix). DNA was extracted from small pieces of pectoral muscle (average 0.05 g) using phenol–chloroform extractions described by Seutin et al. (1993).

PCR amplifications and sequencing. A DNA sequence of 681 base pairs (bp), corresponding to approximately 45% of the COI gene, was amplified by the

<table>
<thead>
<tr>
<th>Species</th>
<th>Family or subfamily</th>
<th>n</th>
<th>No. of differences between samples; TS + TV</th>
<th>Location</th>
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<tr>
<td><em>Troglytes aedon</em></td>
<td>Troglodytidae</td>
<td>1</td>
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<td>St. Lucia, West Indies</td>
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<tr>
<td><em>Toxostoma rufum</em></td>
<td>Mimidae</td>
<td>1</td>
<td>na</td>
<td>Peru</td>
</tr>
<tr>
<td><em>Cinclocerthia ruficauda</em></td>
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<td>5 + 0</td>
<td>Louisiana; U.S.A.</td>
</tr>
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<td><em>Vireo flaviciridis</em></td>
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<td>3 + 1</td>
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</tr>
<tr>
<td><em>Vireo altifrons</em></td>
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<td>St. Vincent, West Indies</td>
</tr>
<tr>
<td><em>Dendroica adalaidae</em></td>
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<td>0 + 0</td>
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<tr>
<td><em>Tangara inornata</em></td>
<td>Thraupinae</td>
<td>2</td>
<td>0 + 0</td>
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<tr>
<td><em>Thraupis episcopus</em></td>
<td>Thraupinae</td>
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<td>na</td>
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<tr>
<td><em>Ramphocelus carbo</em></td>
<td>Thraupinae</td>
<td>2</td>
<td>0 + 0</td>
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<tr>
<td><em>Rhodinocichla rosea</em></td>
<td>Cardinalinae</td>
<td>2</td>
<td>0 + 0</td>
<td>Prov. de Panama; Panama</td>
</tr>
<tr>
<td><em>Saltator albicollis</em></td>
<td>Cardinalinae</td>
<td>1</td>
<td>na</td>
<td>St. Lucia, West Indies</td>
</tr>
</tbody>
</table>


* na: not applicable.
polymerase chain reaction (PCR) using primers COIa and COIF described by Palumbi et al. (1991). Amplifications were carried on for 25 cycles with Perkin-Elmer AmpliTaq DNA polymerase. In each cycle, samples were denatured at 94°C for 45 s, annealing was at 55°C for 45 s, and extension was at 72°C for 60 s. Cycling was preceded by DNA denaturation at 94°C for 3 min and was followed by a 5-min extension at 72°C. Amplification products were eluted in water from low-melting point agarose gels using the GeneClean procedure.

We directly sequenced the amplification products using both manual and automated sequencing techniques. The sequencing primers were those used for the primary amplifications. For manual sequencing, we used the dideoxynucleotide chain termination method with double-stranded DNA as template, following the protocol of Palumbi et al. (1991). Radiolabeled products were run into 6% LongRanger gels at 30 W for 2 to 5 h. In turn, the gels were dried under vacuum and exposed to Kodak XAR films for 24 to 120 h. For automated sequencing, we used the cycle-sequencing method with dye-terminator chemistry. Cycle-sequencing amplifications were carried out in a Perkin-Elmer Thermal Cycler Model 9600, following the protocols provided by the manufacturer (ABD; Applied Biosystems Division of Perkin-Elmer) except that the final reaction volume was brought down to 13.3 µl. Sequencing reactions were run in an ABD Model 373A automated sequencer after unincorporated terminators had been removed using CentriSep columns.

For two samples (one Thraupis episcopus and one Saltator albicollis), we used both sequencing techniques and the resulting sequences were identical. We have found similar consistency between manual and automated sequencing for additional birds in another study (Seutin and Bermingham, in preparation).

Sequence analyses. We read sequences from autoradiographic films independently of one another and compared them to the homologous chicken sequence (Desjardins and Morais, 1990) using the MacVector computer package (version 4.1; I.B.I., 1993). Automatically collected sequence files from the ABI automated sequencer were imported into MacVector and similarly compared to the chicken sequence. Insertions, deletions, and mutations yielding significant amino acid substitutions revealed in these alignments were double-checked.

Bias in nucleotide usage at each codon position was summarized using the C index (e.g., Irwin et al., 1991),

\[ C = \left(\%\right) \sum_{i=1}^{4} \left| c_i - 0.25 \right|, \]

where \( c_i \) is the frequency of the \( i \)th base in the sequence. Calculations were based on nucleotide frequencies averaged across all samples. To quantify intersequence variation in nucleotide usage, we calculated the coefficient of variation (CV) of the proportion of each base in the different sequences for each of the three codon positions. CV of the four nucleotides at a codon position were then averaged to quantify overall intersequence variability by position.

To assess whether groups of sequences shared distinct compositional biases, we used both the GC-tree method proposed by Lockhart et al. (1994) and a principal component analysis (PCA) approach. The analyses focused only on third codon positions (i.e., where intersequence variation in bias was most extensive). The PCA was based on the correlation—not the covariance—matrix, allowing each original variable (i.e., each base) to contribute relatively equally to the analysis (Legendre and Legendre, 1983). This was deemed appropriate since small variations in usage of rarely used nucleotides can have as much an effect on phylogenetic analyses as large differences at frequently used bases. Scores for each original object (i.e., sequences) in the reduced space were calculated and objects were plotted in the bivariate space of the first two principal axes.

Phylogenetic analyses. Because levels of divergence between conspecific sequences were very small compared to those between taxa (Table 1), we included only one sequence per species in phylogenetic analyses. Deleted sequences were chosen by the flip of a coin. The presence of phylogenetic information in the data set was evaluated using the tree-skewness statistic, \( g_1 \), as suggested by Hillis and Huelsenbeck (1992). Calculations were performed using PAUP (version 3.1.1; Swofford, 1993), and statistical significance was judged using Table 2 of Hillis and Huelsenbeck (1992), which is apparently appropriate even for the analysis of strongly biased sequences (p. 193).

We used the program DNAML (version 3.5; Felsenstein, 1993) to produce maximum likelihood (ML) estimates of the phylogeny of sequences. In different analyses, the transition to transversion (TS:TV) ratio was set to 2, 4, and 15. A ratio of 2 is close to the average value observed in the present data set between distant comparisons. At the other extreme, 15:1 is a good approximation of the ratio at which the two types of substitutions occur at presumably neutral fourfold degenerate sites in avian mtDNA (Seutin and Bermingham, in preparation). Two categories of nucleotide sites were defined, one for synonymous and one for nonsynonymous positions. We followed the usual convention (Nei and Gojobori, 1986) and estimated the number of synonymous sites as being equal to the number of fourfold degenerate sites plus one-third of the number of twofold sites. Under this convention, 22% of the sequence positions in our alignment were synonymous. The initial slope of the bivariate distribution of pairwise estimates of divergence at synonymous versus nonsynonymous sites (Fig. 2) suggested that substitu-
tion rate at synonymous positions was 250 times faster than that at nonsynonymous sites. In all ML searches, taxa were introduced at random, and empirical base frequencies were used. ML trees produced, assuming different TS:TV ratios, were compared using a \( \chi^2 \) test with one degree of freedom on twice the difference in log-likelihood of the two trees. Felsenstein (1988) suggested that this is a conservative statistical test of the difference between two ML trees.

For distance-based analyses, we used Kimura's (1980) model to quantify divergence between sequences, considering only TVs. A tree summarizing the matrix of pairwise distances was constructed using the neighbor-joining (NJ) algorithm and was evaluated using the bootstrap procedure (500 replicates) implemented in the MEGA package (Kumar et al., 1993).

In cladistic analyses, various weighting schemes for TSs and TVs were used: only TVs were considered (TV parsimony), TSs were weighted four times less than TVs at all positions (weighted parsimony), and TSs were weighted four times less than TVs at first and second codon positions and ignored at first positions of leucine codons and at all third positions (mixed-weighting parsimony). In the latter analyses, stepmatrices were built so that a TV at a third position had the same weight as a TS at another position (except for first positions of leucine codons). Most-parsimonious (MP) cladograms were generated using the branch-and-bound search strategy in PAUP. Bootstrap assessments of the phylogenetic hypotheses were based on 500 replicates of heuristic searches, with a single random addition of samples and a full branch-swapping (using the TBR procedure) in each search.

We used the paired-sites test based on maximum likelihood to statistically evaluate whether MP cladograms found in different searches were different from one another (Kishino and Hasegawa, 1989). The program DNAML performs this test. The topology of the cladograms of interest was given, but branch lengths were assessed by the program assuming the existence of two categories of sequence sites, as defined above, and a TS:TV ratio of 4. Similarly, this test was used to compare the topology of the best ML tree to trees in which Rhodinocichla had been constrained a priori to be a mimid.

**RESULTS**

**COI Sequences, Base Composition, Variability, and Pattern of Substitution**

Between 578 and 630 bp of homologous DNA sequence of the COI gene were obtained from 21 individual samples representing 13 species: Rhodinocichla rosea, ten possible relatives, and two outgroups (Table 1; Fig. 3). The sequences were deposited in GenBank under accession numbers U91949 to U91961. We observed no insertions or deletions in comparison to the homologous portion of the chicken COI gene (Desjardins and Morais, 1990). The analyzed sequence encodes 6 of the 12 transmembrane helices identified in subunit I of bovine heart cytochrome c oxidase (Tsukihara et al., 1996). These helices are apparently conserved in all aa3-type cytochrome c oxidases characterized thus far (Capaldi, 1990; Hosler et al., 1993).

Figure 4 summarizes the base composition of the 13 sequences presented in Fig. 3 and provides the index of nucleotide bias, \( C \), indicating differences in the intensity of bias at each position. There was a strong bias against the use of guanine and thymine residues at third codon positions, and one in favor of thymine at second positions. Base composition at first positions was relatively unbiased.

Intersequence variation in the use of each nucleotide was calculated as the coefficient of variation (CV) of their frequency in the 13 sequences. Average CV was 3.2% for first codon positions, 1.7% for second positions and 20.9% for third positions. Because variation was extensive at third positions, we were interested to see if groups of sequences shared distinct biases at that position. The "GC tree" (Lockhart et al., 1994) indicated the following relationships: ((Tangara, (Toxostoma, Cinclocerthia), (Saltator, (((Mimus, Troglodytes), (Turdus, Thraupis)), Ramphocelus), (Rhodinocichla, Dendroica))), Vireos). This tree has little relationship to phylogenetic trees produced using ML, distance, and parsimony approaches (see below). We also investigated base composition similarities among sequences by principal component analysis (PCA). The first two axes of the PCA space explained 84.8% of the total variance in nucleotide frequency among sequences.
There were no obvious clusters of taxa in this reduced space (Fig. 5). There were 183 variable positions in the alignment of 13 sequences (Fig. 3), 135 of which were potentially phylogenetically informative. Few first and second codon positions were variable (8.1 and 0.9%, respectively). Most changes at first positions (12 of 16) were silent TSs at triplets coding for leucine; three TSs implied replacement of amino acids with similar physico-chemical properties (Ile-Val; Ala-Thr; Ala-Ile) as did one TV (Ala-Ser). The two substitutions recorded at second codon positions were TSs leading to amino acids differing in properties (Asp-Val; Gln-Glu). The majority of changes at these positions (124 of 135) were silent. There were 16 amino acid substitutions, only seven of which were phylogenetically informative. These were substitutions at first positions: Ile-Ala; Val-Leu; Ala-Leu; Thr-Glu; Thr-Leu; Gln-Glu; Glu-Glu; and one TV (Val-Leu). The remaining substitutions were at second positions: Asp-Val; Gln-Glu; Asp-Glu; Glu-Leu; Glu-Glu; and two TV (Val-Leu; Asp-Glu).
acid replacements of little consequence (Ala or Thr to Ile; Met to Thr). Two TVs at third codon positions also resulted in conservative amino acid replacements (Ile-Met; Gln-His). In total, four of the seven amino acid replacements were in transmembrane helices which composed 74% (157 of 211) of the polypeptide sequence surveyed (Tsukihara et al., 1996). Two variable positions were in cytoplasmic interhelix loops, and one in a periplasmic helix. All polymorphic amino acid sites identified in this study had previously been found to be variable in comparisons across eukaryotes (Capaldi, 1990).

A large proportion (74.7%) of the third codon positions was variable. Patterns of nucleotide substitutions...
FIG. 3—Continued

at that position were investigated by plotting the proportion of transitional and transversional differences observed in pairwise comparisons of sequences against one another (Fig. 6). Under neutral evolution, the maximum divergence expected in such comparisons (i.e., the ultimate saturation level) is given for TSs by $2(\pi_A \pi_C + \pi_T \pi_T)$, and for TVs by $2(\pi_A + \pi_C)(\pi_C + \pi_T)$,

where $\pi_j$ are equilibrium frequencies of the $j$ bases (Kondo et al., 1993).

Figure 6 indicates that saturation level for TSs was slightly above 20%. That this value was higher than the one predicted under neutral evolution (i.e., 14.6%; using observed average base frequencies as representing stationary frequencies in the formula of Kondo et al.)
Rhodinocichla IS AN EMBERIZID

<table>
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<tr>
<th></th>
<th>First Position</th>
<th>Second Position</th>
<th>Third Position</th>
</tr>
</thead>
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<tr>
<td>C</td>
<td>0.071</td>
<td>0.221</td>
<td>0.431</td>
</tr>
<tr>
<td>G</td>
<td>0.500</td>
<td>0.500</td>
<td>0.500</td>
</tr>
<tr>
<td>A</td>
<td>0.221</td>
<td>0.221</td>
<td>0.221</td>
</tr>
<tr>
<td>T</td>
<td>0.431</td>
<td>0.431</td>
<td>0.431</td>
</tr>
<tr>
<td>C</td>
<td>0.071</td>
<td>0.221</td>
<td>0.431</td>
</tr>
</tbody>
</table>

The saturation level for third position TVs cannot be calculated directly from the formula of Kondo et al. (1993) since many such substitutions result in amino acid replacements and are probably not neutral. Looking exclusively at presumably neutral fourfold degenerate sites, we obtained a TV saturation level of 47.5%. Since fourfold sites represented on average 52% of the third positions in our sequences, saturation of fourfold sites corresponded to transversional divergence of at least 24.9% at third positions. This is approximately the value seen in comparisons of passerine and chicken sequences (Fig. 6). The values observed in our compari-

![Graph](image-url)
sons of passerine sequences were much lower (maximum 18.7%), indicating that even the most readily substitutable portion of third codon positions was not saturated with TVs in our study group.

**Relationships of Rhodinocichla**

All the phylogenetic reconstructions that we produced concluded that *Rhodinocichla* was an emberizid and not a mimid. This outcome was seen, for example, in the ML tree found assuming a TS:TV ratio of 4 (Fig. 7, left). The TS:TV = 4 tree had a significantly higher log-likelihood than trees produced when a ratio of 2 or 15 was assumed (comparing trees using ratios of 2 and 4: $\chi^2 = 29.25, P = 0.000$; trees using ratios of 4 and 15: $\chi^2 = 84.61, P = 0.000$). Emberizine sequences formed a monophyletic group which included *Rhodinocichla rosea* in all three ML trees. Thus, our conclusion that *Rhodinocichla* is an emberizid holds across a wide range of TS:TV ratios.

The NJ tree (not shown) summarizing the matrix of Kimura’s (1980) TV distances had the same topology as the best ML tree (Fig. 7, left), except that emberizine sequences were related as follows: ((Dendroica (Tangara (Thraupis, Saltator))) (Rhodinocichla, Ramphocelus)). A bootstrap analysis provided relatively good support for the monophyly of emberizids including *Rhodinocichla* and of mimids without *Rhodinocichla* (Table 2).

Cladistic analyses revealed several equally parsimonious topologies: five cladograms of 145 steps under TV parsimony, one of 948 steps under weighted parsimony, and five of 161 steps under mixed-weighting parsimony (individual cladograms not shown). In total, searches under the three weighting schemes produced seven distinct topologies, and paired-sites log-likelihood tests suggested that all of them were statistically equivalent estimates of the phylogeny of the studied sequences (largest log-likelihood difference = 0.328 S.D.). A majority consensus tree of the seven cladograms (Fig. 7, right) indicated once more that *Rhodinocichla* belongs to the emberizid radiation. Bootstrap analysis provided little support for the monophyly of emberizids, including *Rhodinocichla*, and of mimids without *Rhodinocichla*, in searches under each weighting scheme (Table 2).

To evaluate quantitatively the likelihood that *Rhodinocichla* was a mimid, we conducted parsimony searches under that constraint. Table 3 shows that under all weighting schemes, the shortest constrained cladograms ($L_{Min}$) were many steps longer than the corresponding unconstrained MP cladograms ($L_{MM}$). In all cases, there was a very large number of more parsimonious networks than the shortest constrained cladograms ($N_{Non-Mimids}$; Table 3). Two distinct topologies were found in constrained searches using different weighting schemes. In both cases, the relationships of mimid sequences were (*Rhodinocichla* (Mimus (Cinclocerthia, Toxostoma))). The log-likelihoods of the two constrained topologies were less than that of the best ML tree (-2965 and -2969 versus -2952), but paired-sites log-likelihood tests indicated that those differences were not statistically significant (log-likelihood differences = 1.828 and 1.433 S.D., $P = 0.067$ and 0.153, respectively).

**Phylogenetic Relationships within the Emberizidae**

Most internal branches in the emberizid clade resolved in ML analyses were very short and not statisti-
Rhodinocichla is an Emberizid

FIG. 7. Phylogenetic analyses of 13 passerines based on partial COI sequences. The maximum likelihood model (left panel; log-likelihood = −2952) assumed two categories of sites (synonymous versus nonsynonymous) and a transition to transversion ratio of 4; branches judged by log-likelihood ratio tests not to be significantly positive in length were not drawn. The parsimony tree (right panel) is a majority consensus tree of the seven distinct topologies found in searches under three weighting schemes (see Materials and Methods); values in circles indicate the frequency at which a specific partition was found across topologies, and branches found in less than 50% of the topologies were not shown.

Saltator albicollis and Thraupis episcopus were identified as sister-taxa in all analyses. The branch leading to this pair was significantly positive in length in the best ML tree and received relatively good support in bootstrap analyses of the NJ tree and two MP searches (Table 2).

In the best ML tree and the consensus of MP cladograms (Fig. 7), Rhodinocichla rosea was found to be the sister taxon of Ramphocelus carbo. Although the branch leading to this pair in the ML tree was significantly positive in length, the relationship was not supported in the bootstrap analysis of the NJ tree (41%) or of MP searches (all <50%).

TABLE 2

Bootstrap Support (in percentage) for Specific Clades in Neighbor-Joining and Parsimony Analyses of the Relationships of 13 Passerine Taxa

<table>
<thead>
<tr>
<th>Analysis*</th>
<th>Emberizidae</th>
<th>(Thraupis + Saltator)</th>
<th>(Turdus + Troglydotes)</th>
<th>Mimidae</th>
</tr>
</thead>
<tbody>
<tr>
<td>NJ</td>
<td>79</td>
<td>78</td>
<td>64</td>
<td>74</td>
</tr>
<tr>
<td>MP-TS0</td>
<td>59.2</td>
<td>72.5</td>
<td>73.1</td>
<td>53.9</td>
</tr>
<tr>
<td>MP-TV4</td>
<td>56.1</td>
<td>50.8</td>
<td>61.8</td>
<td>55.7</td>
</tr>
<tr>
<td>MP-mixed</td>
<td>47.2</td>
<td>81.1</td>
<td>67.0</td>
<td>68.3</td>
</tr>
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</table>

Note. The neighbor-joining tree was based on a matrix of pairwise Kimura's (1980) transversion distances. Three parsimony analyses were performed under different weighting schemes for transition and transversion substitutions. Emberizidae here include the genera Dendroica, Tangara, Thraupis, Ramphocelus, Saltator, and Rhodinocichla; Mimidae include Mimus, Toxostoma, and Cinclocerthia.

* NJ: neighbor-joining analysis; MP-TS0: parsimony analysis, transitions weighted 0; MP-TV4: parsimony analysis, transversions weighted four times more than transitions; MP-mixed: parsimony analysis, TS0 weighting applied to first codon positions of leucine codons and all third positions, TV4 weighting applied to other positions.

TABLE 3

Summary of Parsimony Analyses of the Relationships of Rhodinocichla rosea

<table>
<thead>
<tr>
<th>Weighting*</th>
<th>$L_{\text{Min}}$</th>
<th>$L_{\text{Mimid}}$</th>
<th>$N_{\text{Non-mimid}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-TS0</td>
<td>145 (5)</td>
<td>150 (1)</td>
<td>590</td>
</tr>
<tr>
<td>MP-TV4</td>
<td>948 (1)</td>
<td>974 (1)</td>
<td>3653</td>
</tr>
<tr>
<td>MP-mixed</td>
<td>161 (5)</td>
<td>167 (2)</td>
<td>928</td>
</tr>
</tbody>
</table>

Note. $L_{\text{Min}}$: length (and number) of the most-parsimonious tree(s) found under different weighting schemes for transition and transversion substitutions. $L_{\text{Mimid}}$: length (and number) of most-parsimonious tree(s) found under the constraint that Rhodinocichla was a Mimidae. $N_{\text{Non-mimid}}$: number of trees shorter than $L_{\text{Mimid}}$.

* Acronyms for weighting schemes defined in footnote of Table 2.
Other Phylogenetic Relationships

The monophyly of mimids excluding *Rhodinocichla* was indicated by the ML analysis (branch length significantly positive) and moderately supported by a bootstrap analysis of the NJ tree (Table 2). It was also indicated by MP analyses but with little bootstrap support (Table 2). In most searches, *Cinclocerthia* and *Toxostoma* were identified as sister taxa.

All the phylogenetic reconstructions that we produced indicated that *Turdus* and *Troglydytes* sequences formed a clade that was the sister group of the mimids. This consistently observed clade was only moderately supported by bootstrap analyses (Table 2) and might be an example of the long-branch attraction phenomenon. DNA–DNA hybridization results suggested different relationships (Sibley and Ahlquist, 1990; see Fig. 1).

### DISCUSSION

We have sequenced approximately 600 bp of the mitochondrially encoded COI gene in a variety of passerine birds (Table 1; Fig. 3). Our primary purpose was to investigate the evolutionary relationships of *Rhodinocichla rosea*, an oscine whose phylogenetic affinities had not been well established. Our study also presents an opportunity to discuss molecular evolution of COI in passerines and to evaluate the potential of this gene for phylogenetic studies of birds.

**Molecular Evolution of COI in Passerine Birds**

Important biases in nucleotide usage have been documented in all vertebrate mtDNA genomes studied thus far (e.g., Wolstenholme, 1992). The bias we have observed in passerine COI (Fig. 4) was typical for a protein-coding gene translated from the H strand of avian mtDNA (Desjardins and Morais, 1990; Edwards *et al.*, 1991; Kornegay *et al.*, 1993; Nunn *et al.*, 1996; Seutin and Bermingham, in preparation). Further, the intensity of the bias at each codon position, as reflected by C-index values (Fig. 4), was very similar to those estimated for passerine, galliformes, and albatrosses *Cytb* sequences (Edwards *et al.*, 1991; Kornegay *et al.*, 1993; Nunn *et al.*, 1996). Thus, with regard to nucleotide bias, COI does not differ from the most commonly used gene in avian molecular systematics.

There has been considerable discussion recently regarding heterogeneity in nucleotide bias across sequences and its influence on phylogenetic inferences. Of primary concern is the fact that convergence at silent positions can lead to the grouping of taxa according to nucleotide bias rather than shared genealogies (e.g., Lockhart *et al.*, 1994). Variation in nucleotide usage among our passerine COI sequences was very low at first and second codon positions, but substantial at third positions (Fig. 4; see Results). This observation probably reflects the reduced selective constraints under which third positions evolve in the mitochondrial genome owing to the degeneracy of the genetic code. Practically, this means that it is at third codon positions that variation in nucleotide bias is likely to present problems in systematic investigations. We propose that a principal component analysis of variation in nucleotide usage is a useful way of summarizing heterogeneity in bias in a set of homologous sequences and of identifying groups of sequences characterized by distinct biases. To our knowledge, ordination methods (e.g., PCA) have not been used before for this purpose although they can identify relationships that might not be revealed by tree-producing analyses (e.g., Sneath and Sokal, 1973; Legendre and Legendre, 1983). In passerine COI, we failed to identify such groups (Fig. 5). Further, a "GC tree" (Lockhart *et al.*, 1994) failed to reveal clusters found in the phylogenetic analyses. Thus, variation in nucleotide usage in our data set should not have confounded our phylogenetic analyses.

COI is the most conservative mitochondrial gene in terms of amino acid evolution (e.g., Desjardins and Morais, 1990; Capaldi, 1990; Hosler *et al.*, 1993). The encoded polypeptide is the largest subunit of the cytochrome *c* oxidase enzyme, and it contains the amino acid ligands for three of the four redox centers of that protein (Tsukihara *et al.*, 1995). Analyses by site-directed mutagenesis have demonstrated that changes at many positions in this subunit significantly disrupt the function of the holoenzyme (reviewed in Hosler *et al.*, 1993). Thus, strict functional constraints might explain why replacement substitutions were rare in the sequences we studied even though the represented taxa have been diverging for as long as 40 MY (Sibley and Ahlquist, 1990). Because few amino acid sites are free to vary in the COI molecule, genetic distance measures using nonsynonymous substitutions will be associated with large error terms and, consequently, will be of limited use for phylogenetic reconstructions.

In contrast to nonsynonymous sites, selective constraints are greatly reduced at synonymous sites because substitutions at these positions are not expressed phenotypically. The bivariate plot of pairwise divergence at synonymous and nonsynonymous sites (Fig. 2) revealed the difference in the frequency of the two types of substitutions in avian COI. For the narrow range of divergence in which synonymous changes were not saturated, the ratio of changes was approximately 250:1 in favor of synonymous substitutions. The synonymous:nonsynonymous ratio has been estimated to vary from 5:1 to 100:1 in other mitochondrial genes representing different vertebrates (Brown and Simpson, 1982; Brown *et al.*, 1982; Irwin *et al.*, 1991; Kondo *et al.*, 1993). Variation in reported ratios resulted partly from comparisons made between sequences of varying degrees of relatedness. However, it is also clear that distinct ratios characterize different mitochondrial genes because rates of silent substitutions vary little among genes in a genome (Sharp and Li, 1989; Nigro *et
Phylogenetic Relationships of \textit{Rhodinocichla} rosea

Phylogenetic analysis of COI sequences using maximum likelihood, distance, and parsimony methods indicated that \textit{Rhodinocichla} rosea is not a mimid, but an emberizid (Fig. 7). This conclusion is robust for three reasons. First, we used an eclectic array of phylogenetic reconstruction techniques, and each method supported our conclusion. Thus, our taxonomic assignment was independent of the assumptions specific to each method (e.g., Friday, 1987). Second, the inclusion of \textit{Rhodinocichla} in the emberizids was unaffected by the weighting scheme applied to different types of substitutions, as long as saturated TSs at third codon positions were given a low weight. Third, cladograms in which \textit{Rhodinocichla} was constrained to be a mimid appeared worse than MP unconstrained cladograms, as judged by the greater length of the former and the existence of very large numbers of cladograms shorter than the best constrained one (Table 3). However, paired-sites likelihood tests (Kishino and Hasegawa, 1989) indicated that the constrained and unconstrained topologies did not represent statistically different phylogenetic hypotheses for the sequences. This result may be explained in part by the conservative nature of the paired-sites test (A. Sidow, in Zimmer et al., 1994).

Our taxonomic assignment of \textit{Rhodinocichla} was based on the study of part of a single gene, the mitochondrially encoded COI gene, and would be strengthened by congruent results from analysis of at least one independent set of genetic markers. The potential problem with a study based on a single nonrecombining sequence is that it provides a gene pedigree which represents only one realization of the multigene process of lineage sorting through an organismal pedigree (e.g., Avise, 1994). Thus, if taxa of interest have not, or have only recently, reached a state of reciprocal monophyly for the gene considered, erroneous conclusions might be drawn from directly inferring the taxa phylogeny from the single gene pedigree. In the present study, we were interested in allocating a taxon to one of two distantly related families, the Mimidae and Emberizidae, which have probably diverged 20–30 MY ago (Sibley and Ahlquist, 1990). Work by Avise and collaborators (e.g., Avise, 1994) has indicated that for rapidly evolving neutral markers such as mtDNA polymorphisms, it is unlikely that two taxa would not have reached a state of reciprocal monophyly after such a long time. This conclusion should hold true even if mtDNA variation is not entirely neutral, provided that the polymorphisms are not subject to stabilizing selection. Thus, we believe that the use of a single gene in this study does not significantly weaken our phylogenetic assignment. Furthermore, as Moore (1995) has shown in his contrast of mitochondrial and nuclear phylogenetic markers, our study represents a pragmatic approach to the \textit{Rhodinocichla} problem.

Although our analysis of COI sequences strongly indicates that \textit{Rhodinocichla} rosea is not a mimid, its position within the emberizids could not be established. The problem stems from the fact that the typical tanagers we studied (i.e., \textit{Tangara}, \textit{Thraupis}, and \textit{Ramphocelus}) did not form a well-supported clade, distinct from the Parulinae and Cardinalinae (Fig. 7). There are two possible reasons for this. First, the tanagers \textit{sensu} A.O.U. (1983) might not form a monophyletic taxon, a possibility that has been raised repeatedly in the past (e.g., Ridgway, 1902; Storer, 1967). DNA–DNA hybridization studies (Bledsoe, 1988; Sibley and Ahlquist, 1990) have indicated that a higher taxon comprising the typical tanagers (\textit{sensu} A.O.U., 1983) and a number of thick-billed species customarily classi-
fied as finches (Emberizinae, Emberizidae; A.O.U., 1983) might form a natural entity. Our data suggested a close relationship between *Saltator albicollis*, which is currently classified as a grosbeak (Cardinalinae, Emberizidae; A.O.U., 1983), and a typical tanager, *Thraupis episopus*. That saltators might be tanagers had been suggested by some authors in the 19th century (e.g., Sclater, 1886). To establish the limits of a monophyletic tanager taxon, it will be necessary to genetically analyze a number of finches (Bledsoe, 1988; Sibley and Ahlquist, 1990), wood-warblers (e.g., *Xenopithex, Micrornis, Granatellus*), grosbeaks (e.g., *Saltator*, *Pitlyus, Caryothraustes*; Demastes and Remsen, 1994; this study), as well as the taxa formerly included in the family Coerebidae (Storer, 1967).

If *Saltator albicollis* is indeed a tanager, our concern about the monophyly of tanagers is limited to the observation that *Dendroica adelaidae* fell among tanagers in some phylogenetic reconstructions. *Dendroica* warblers have never been suggested to be tanagers and the existence of the Parulinae and Thraupinae as distinct taxa has never been seriously questioned. If the two groups are indeed distinct monophyletic lineages, the fact that they failed to resolve as distinct entities in many analyses is probably due to the limited informativeness of our sequence data. Indeed, tree-skewness statistics (Hillis and Huelsenbeck, 1992) indicated the presence of phylogenetic information in the complete data set, but suggested that little of it was found in the set of emberizid sequences (for TV parsimony data: $g_1 = -0.653$, 45 variable sites, $P > 0.05$; for weighted parsimony data: $g_1 = -0.273$, 151 variable sites, $P > 0.05$; for mixed-weighting parsimony data: $g_1 = -1.088$, 151 variable sites, $P < 0.05$). The low information content of the emberizid sequences was also reflected by the short internal branches in the corresponding portion of ML and NJ trees (Fig. 7). Perhaps the radiation of emberizids has been explosive with little time for the accumulation of diagnostic molecular traits between cladogenetic events. DNA–DNA hybridization data (Bledsoe, 1988; Sibley and Ahlquist, 1990) also suggested a rapid diversification of the family. As we have established above, however, some of our difficulty in reconstructing emberizid relationships certainly is due to the nature and low number of phylogenetically informative characters in the roughly 600 bp of COI analyzed. A thorough study of emberizid relationships will also require a better sampling of the group-specific diversity. We are currently conducting such a survey using rapidly evolving mitochondrial sequences.

In conclusion, our identification of *Rhodinocichla rosea* as an emberizid indicates that many of the phenotypic characteristics that suggested its phylogenetic relationship with mimids have evolved through convergence. It would now be useful to follow an integrated approach linking the behavioral, morphological, and myological peculiarities of this distinctive species to its unique natural history characteristics. We also hope that better resolution of phylogenetic relationships within an enlarged tanager higher taxon will help identify factors that have led to the impressive tanager radiation (see Storer, 1967).

### APPENDIX 1: Information Regarding Tissue Samples

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Field/Voucher No.</th>
<th>Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troglydotes aedon</td>
<td>B</td>
<td>D-TAE1</td>
<td>STRI</td>
</tr>
<tr>
<td>Turdus nudigenis</td>
<td>B</td>
<td>M-TNU1; SL-TNU1</td>
<td>STRI</td>
</tr>
<tr>
<td>Minus longicaudatus</td>
<td>S</td>
<td>LSUMZ 3810</td>
<td>LSU</td>
</tr>
<tr>
<td>Taxostoma rufum</td>
<td>S</td>
<td>LSUMZ 0490</td>
<td>LSU</td>
</tr>
<tr>
<td>Cinclorhynchus ruficuia</td>
<td>B</td>
<td>CRU01; CRU011</td>
<td>STRI</td>
</tr>
<tr>
<td>Vireo flavoaviridis</td>
<td>B</td>
<td>VFL1; VFL2</td>
<td>STRI</td>
</tr>
<tr>
<td>Vireo altiroquos</td>
<td>B</td>
<td>D-VAL8; D-VAL11</td>
<td>STRI</td>
</tr>
<tr>
<td>Dendroica adelaidae</td>
<td>S</td>
<td>SL-DAD5; SL-DAD8</td>
<td>STRI</td>
</tr>
<tr>
<td>Tangara inornata</td>
<td>S</td>
<td>GS-PC88; GS-PC93</td>
<td>ANSP</td>
</tr>
<tr>
<td>Thraupis episopus</td>
<td>S</td>
<td>V-TEP1</td>
<td>STRI</td>
</tr>
<tr>
<td>Ramphocelus carbo</td>
<td>B</td>
<td>V-RC1; V-RCA2</td>
<td>STRI</td>
</tr>
<tr>
<td>Rhodinocichla rosea</td>
<td>S</td>
<td>GS-PAA5; GS-PAA1</td>
<td>ANSP</td>
</tr>
<tr>
<td>Saltator albicollis</td>
<td>B</td>
<td>SAL003</td>
<td>STRI</td>
</tr>
</tbody>
</table>

* a B, biopsy; S, tissues and voucher specimen.
* b ANSP, Academy of Natural Sciences, Philadelphia; LSU, Louisiana State University Museum of Natural Science; STRI, Smithsonian Tropical Research Institute.

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### REFERENCES


Rhodinocichla is an Emberizid