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A ROBUST PHYLOGENY OF THE OROPENDOLAS: POLYPHYLY REVEALED BY MITOCHONDRIAL SEQUENCE DATA

J. JORDAN PRICE[1] AND SCOTT M. LANYON

James Ford Bell Museum of Natural History, University of Minnesota, 1987 Upper Buford Circle, St. Paul, Minnesota 55108, USA

ABSTRACT.—We present a robust, fully resolved phylogeny for the oropendolas that will serve as a basis for comparative studies in this group. We sequenced 2,011 base pairs (bp) of the mitochondrial cytochrome-*b* and ND2 genes from 22 individuals to reconstruct relationships between recognized species and subspecies and to assess variation within polytypic taxa. A single phylogenetic tree was produced despite the use of a wide range of weighting schemes and phylogenetic reconstruction methods. Our data provide strong evidence that oropendolas are polyphyletic, with two distinct groups within a larger clade of oropendolas and caciques. We confirm the monophyly of recognized species, but indicate that some within-species relationships do not conform to recognized subspecies limits. Our findings thus demonstrate the importance of including multiple exemplars from each taxon of interest. The two genes provided complimentary and equally effective phylogenetic information for comparisons within the oropendolas, but exhibited lower resolution in comparisons above the species level. *Received 4 April 2001, accepted 7 December 2001.*

RESUMEN.—Presentamos una filogenia robusta y completamente resuelta para las oropéndolas que servirá como base para estudios comparativos en este grupo. Secuenciamos 2,011 pares de bases de los genes mitocondriales citocromo-*b* y ND2 de 22 individuos para reconstruir las relaciones entre las especies y subespecies reconocidas y para evaluar la variación al interior de taxa politípicos. Se obtuvo un único árbol filogenético a pesar de haber utilizado una amplia variedad de esquemas de pesaje y métodos de reconstrucción filogenética. Nuestros datos proveen evidencia fuerte que indica que las oropéndolas son polifiléticas, con dos grupos distintivos dentro de un clado más grande de oropéndolas y caciques. Confirmamos la monofilia de las especies reconocidas, pero indicamos que algunas relaciones al interior de especies no concuerdan con los límites subespecíficos reconocidos. Por lo tanto, nuestros hallazgos demuestran la importancia de incluir múltiples ejemplares de cada taxon de interés. Los dos genes suministraron información filogenética complementaria e igualmente efectiva para comparaciones dentro de las oropéndolas, pero mostraron menor resolución en comparaciones por encima del nivel de especie.

THE OROPENDOLAS (genera *Psarocolius*, *Gymnostinops*, and *Ocyalus*; Sibley and Monroe 1990) provide some of the most extreme examples of polygynous breeding, sexual size dimorphism, and vocal display complexity known in birds (Orians 1985, Robinson 1986, Webster 1992, Jaramillo and Burke 1999). These large, colonial-nesting members of the New World blackbirds (Icterini) range in characteristics from the Casqued Oropendola (*Psarocolius oseryi*), in which males consort with only a few females and produce relatively simple courtship displays (Leak and Robinson 1989, Ridgely and Tudor 1989), to the Montezuma Oropendola (*Gymnostinops* [=*Psarocolius*] *montezuma*), in which males defend large harems and perform elaborate display songs that almost defy description (Webster 1994, Howell and Webb 2000). This group thus provides excellent potential subjects for evolutionary biologists, especially those interested in the processes and evolutionary consequences of sexual selection. Several comparative studies have included oropendolas, including studies of size dimorphism (Webster 1992) and mating systems (Robinson 1986). However, phylogenetic relationships among oropendola taxa are poorly understood, which limits the abilities of researchers to apply comparative methods effectively (Brooks and McLennan 1991, Harvey and Pagel 1991, Martins 1996).

Systematic studies of this group have been limited, but generally agree on a close relation-

[1] E-mail: price037@tc.umn.edu

ship between the oropendolas and the caciques (*Cacicus*; e.g. Sibley and Ahlquist 1990). Although differing in some characteristics, especially body size (oropendolas are among the largest of all passerines), these two groups share a variety of morphological and behavioral features (Beecher 1951, Orians 1985, Skutch 1996, Jaramillo and Burke 1999). Lanyon and Omland (1999) included four oropendolas and four caciques in a molecular study of blackbird relationships using cytochrome-*b* sequence data. That analysis provided strong evidence for a monophyletic lineage consisting of both the oropendolas and caciques, but was not able to demonstrate the monophyly of either group. Freeman and Zink (1995) included four oropendolas and five caciques in a study using mitochondrial DNA restriction sites. Their data resolved two separate groups of oropendolas within a larger oropendola–cacique assemblage, which suggests that the oropendolas are not monophyletic. Although neither study resolved relationships among all taxa included, they provide two testable phylogenetic hypotheses: the existence of an oropendola–cacique clade and polyphyly of the oropendolas.

In addition to addressing phylogenetic relationships among genera and species, our study examines relationships below species level by including multiple subspecies and geographic variants, which allows us to address three further objectives. First, we are able to test the monophyly of species, and in some cases that of subspecies. Verifying monophyly at every taxonomic level is a crucial component of reliable tree-building and, hence, proper application of the comparative approach (Brooks and McLennan 1991, Lanyon 1993). If a taxon is polyphyletic or paraphyletic, its hypothesized position in a phylogenetic tree can be substantially altered depending on what representative lineage is sampled (Lanyon 1994), which can lead to erroneous conclusions about character evolution. Second, multiple within-species sampling has the potential for enhancing the resolution and reliability of our phylogenetic hypotheses. Increasing the number of taxa sampled in a group of interest has been suggested as an effective means for producing well-resolved and strongly supported trees (Hillis 1996, Graybeal 1998, Omland et al. 1999), mostly because such additions tend to subdivide long branches (Felsenstein 1978). Third, subspecies relationships in several oropendola taxa are controversial and would benefit from formal systematic study (Jaramillo and Burke 1999). For example, Blake (1968) divides the Russet-backed Oropendola (*Psarocolius angustifrons*) into seven geographically distinct subspecies, whereas Sibley and Monroe (1990) divide it into only two: *P. a. angustifrons*, which includes two of Blake's (1968) subspecies, and *P. a. alfredi*, which includes five. Some authors have further suggested dividing this complex into two species (e.g. Ridgely and Tudor 1989, Jaramillo and Burke 1999); however, the proposed phylogenetic break between those taxa is different than that defined by Sibley and Monroe (1990). By constructing mitochondrial DNA (mtDNA) phylogenies using samples from multiple subspecies and populations, we hope to eliminate some of the uncertainty about within-species relationships and taxonomic limits in this complex, as well as among other oropendola taxa. Furthermore, because many characteristics of interest to evolutionary biologists vary between subspecies, or even between populations (e.g. body size, song patterns), the phylogenetic tree of oropendola relationships presented here can serve as a useful foundation for comparative studies in this assemblage.

METHODS

Taxa sampled.—We obtained samples from 25 oropendola individuals for our study, chosen to represent variation within and between 8 of the 11 species of oropendola recognized by Sibley and Monroe (1990). To ensure adequate sampling of within-species diversity, we included multiple subspecies, samples from geographically distant collecting localities, or both, for six of the species. For three taxa, we also analyzed two individuals from the same locality to test for within-population diversity; however, none differed by more than one base substitution so only one sample per locality is presented here. Two species, *Psarocolius oseryi* and *Ocyalus latirostris*, have relatively limited ranges and no described subspecies, so each was represented by a single individual. Throughout this article, we follow the species and genus nomenclature of Sibley and Monroe (1990), because it is the most geographically comprehensive and widely available recent checklist. Where appropriate, however, we use the subspecies names of Blake (1968), because few subspecies are listed in Sibley and Monroe (1990). The Appendix lists sample sources, voucher information, and collecting localities for all specimens used in the study.

For outgroup taxa we chose two caciques, *Cacicus melanicterus* and *C. solitarius*, as well as one representative from each of the other four major clades within the Icterini (Lanyon and Omland 1999). The grackles and allies were represented by *Agelaius phoeniceus*, the orioles by *Icterus galbula*, the meadowlarks and allies by *Sturnella neglecta*, and the monotypic cup-nesting cacique clade by its one member, *Amblycercus holosericeus*. (Although referred to as a cacique, this latter species has distant relationships to other icterines, including members of the genus *Cacicus*; see Lanyon and Omland 1999). Because past studies (e.g. Sibley and Ahlquist 1990, Freeman and Zink 1995, Lanyon and Omland 1999, Klicka et al. 2000) have placed caciques and oropendolas in the same clade within the blackbirds but have not demonstrated monophyly in either, we chose to separate our outgroup taxa into two categories for our analyses. The two caciques were treated as potential ingroup taxa, whereas the remaining four more distant icterine taxa were designated as the definitive outgroup. In that way we were not dependent on an assumption of oropendola monophyly.

DNA sequencing.—We sequenced two mitochondrial genes for our study, cytochrome *b* (cyt *b*) and ND2, previously shown to be effective for resolving relationships within blackbirds (Johnson and Lanyon 1999, Lanyon and Omland 1999, Omland et al. 1999, Klicka et al. 2000). These genes differ in evolutionary rate (Hackett 1996), but those rate differences are not always comparable across lineages (Johnson and Sorenson 1998). Besides providing more characters for phylogenetic analysis, and thus potentially enhancing tree resolution (e.g. Johnson and Lanyon 1999), sequencing two separate gene regions allowed us to compare their patterns of sequence evolution in this avian group and enabled us to assess their relative utility for resolving relationships at different taxonomic levels.

We extracted total genomic DNA from tissue samples using standard phenol chloroform protocols (Sambrook et al. 1989), except for samples from the Smithsonian Tropical Research Institute (see Appendix), which were obtained as lyophilized DNA. We amplified specific, double-stranded fragments of the mitochondrial genome using pairs of primers spanning 926 bp of the cyt-*b* gene and spanning 1,098 bp of ND2, and then produced and sequenced single-stranded amplification products using those primers plus others. Primers used for cyt *b* were L14841 (Kocher et al. 1989; B1), H15149 (Kocher et al. 1989; B2), L15042 (Lanyon 1994; B3), H15767 (Lanyon 1994; B4), and L15243 (Lanyon 1994; B5). ND2 primers used were L5215 (Hackett 1996), L5758 (Johnson and Sorenson 1998), H5776I (Omland et al. 1999), and H6313 (Johnson and Sorenson 1998).

We performed PCR in 50 μL reaction volumes using 0.75 μL Thermo flavus polymerase (Epicentre Technologies), 3 μL of 10 μmol solution of each primer, 4 μL of 25 μmol $MgCl_2$, 1 μL PCR nucleotide mix, 2.5 μL of 25× reaction buffer, between 1 and 4 μL of total genomic DNA extracts, and distilled water. We used a Thermolyne Amplitron II to perform the reactions. A typical amplification involved 35 cycles of denaturation at 93°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min, followed by a final 10 min extension at 72°C and a hold at 4°C. PCR products were cleaned using a Qiagen PCR purification kit following the manufacturer's protocols. We performed sequencing reactions using an ABI Prism BigDye Terminator Cycle Sequencing Kit with manufacturer's protocols and the above given primers, so that specific regions of each gene were sequenced in both directions. Those products were purified using Centrisep columns packed with 0.05 g Sephadex (Sigma) in 0.8 mL water, dried in a Centrivap vacuum concentrator, and sequenced on an ABI 310 automated sequencing machine. We aligned the resulting chromatograms of complementary strands and confirmed them using SEQUENCHER sequence analysis software (Gene Codes Corporation, Ann Arbor, Michigan). Very few peaks were obscured or difficult to read; only 4 nucleotides in both gene sequences across all taxa were scored as ambiguous. In total, we obtained 920 bp of sequence for cyt *b* and 1,091 bp of sequence for ND2 for each sample, including the two caciques and *Amblycercus holosericeus*. Those sequences have been deposited in GenBank under the accession numbers AF472362 to AF472411. Sequences for the other outgroup taxa were obtained from GenBank from the study of Klicka et al. (2000; GenBank accession numbers AF290127, AF290130, AF290134, AF290164, AF290167, AF290173).

Analysis of sequence data.—Aligned sequences were imported into PAUP* (version 4.0b, Swofford 2000) for phylogenetic analysis. We compared effectiveness of each mitochondrial gene for resolving phylogenetic relationships in a number of ways. We examined the nature of base substitutions in the two gene regions by calculating the proportion of base positions in each that were variable and potentially phylogenetically informative, and then comparing those using a *z*-statistic approximation (Milton and Arnold 1990). We compared evolutionary rates in the two genes by plotting pairwise percentage sequence divergences in ND2 against those in cyt *b*. To assess potential for saturation due to multiple substitutions in each gene, we calculated the number of transitions and transversions at each codon position in each gene region for all pairwise comparisons, and then plotted those character transformations against total percentage sequence divergence for each type of change. We also reconstructed transformations in each gene on the combined-data tree by finding the shortest tree using equally weighted parsimony and then reconstructing transitions and transversions from both data sets onto that topology using MAC-

CLADE 4.0 (Maddison and Maddison 2000). To determine if cyt *b* and ND2 contain similar phylogenetic signal, and thus whether combining the two gene regions in our analyses is justified (Bull et al. 1993), we performed a partition homogeneity test in PAUP* (Farris et al. 1994, Swofford 2000) using the two genes as partitions under equal weighting. Because that test revealed no significant incongruence, we combined those sequences in our analyses of oropendola relationships.

Transitions and transversions tend to accumulate at different rates (e.g. Hackett 1996, Griffiths 1997), and so are not expected to be equally phylogenetically informative. We explored sensitivity of our tree topology to differential weighting of transitions and transversions by using a range of weighting schemes in a parsimony analysis of our combined data set. Those included equal weighting (1:1) and weighting of transversions over transitions by 2:1, 4:1, 6:1, 8:1, 10:1, and 15:1. We also performed heuristic searches using both genes independently under each weighting scheme to investigate potential sources of conflict in our data set which might have resulted in low resolution or support in the combined-data tree. To estimate degree to which the results of those analyses were dependent on character composition, we conducted full heuristic bootstrap analyses (Felsenstein 1985) for each weighting scheme, using both genes separately and in combination, with 1,000 replicates. Likewise, to assess the degree to which topology was dependent on taxonomic composition, we performed a jackknife analysis (Lanyon 1985) on the most parsimonious unweighted combined-data tree. To evaluate nodal support for that tree, we calculated the decay index (Bremer 1994) using AUTODECAY 4.0 (Eriksson 1999).

To test whether tree topology was robust with respect to alternative tree-building methodologies, we used several additional algorithms in PAUP*. For a maximum-likelihood analysis, we explored which of 56 possible models of sequence evolution best fit our data using likelihood-ratio tests and the Akaike Information Criterion (AIC) in MODELTEST (version 3.0, Posada and Crandall 1998). Those tests selected two models. The likelihood-ratio tests selected the transversional model with invariant sites and with rates at variable sites following a gamma distribution (TVM + I + G, proportion of invariable sites = 0.5941, gamma distribution shape parameter = 2.1287) and the AIC selected the general time-reversible model with invariant sites and with gamma distributed rates at variable sites (GTR + I + G, proportion of invariable sites = 0.5990, gamma distribution shape parameter = 2.2834; Rodriguez et al. 1990), both with substitution rates and base frequencies estimated from the data set. We used those models in two separate maximum-likelihood tests. We also constructed a neighbor-joining tree using Kimura two-parameter genetic distances (Kimura 1980).

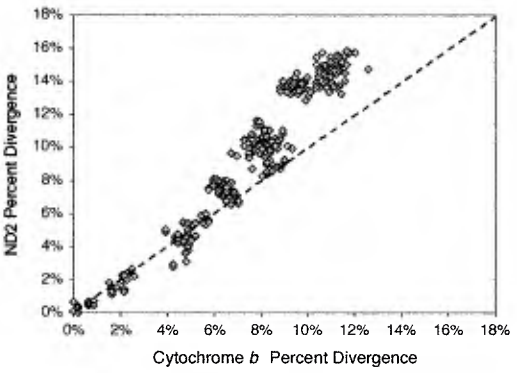

FIG. 1. Plot of percentage sequence divergence in ND2 versus cytochrome *b* in pairwise comparisons of all sequences. The dashed line corresponds to equal divergence rates in the two genes. ND2 appears to accumulate changes more rapidly than cyt *b* at divergences >6%.

RESULTS

Comparison of genes.—ND2 was more variable than cyt *b* for the gene regions sequenced. Of the 1091 bp of ND2 sequence, 385 (35.3%) were variable and 271 (24.8%) were potentially phylogenetically informative. Of the 920 bp of cyt *b*, 279 (30.3%) were variable and 181 (19.7%) were potentially informative. Most of those variable sites were third codon positions (72.2% in ND2 and 81.1% in cyt *b*). These differences in variability were statistically significant as measured by the *z*-statistic approximation ($P < 0.05$) and were reflected in the overall faster rate of evolution in ND2 than in cyt *b* (Fig. 1). The two genes showed similar rates of divergence for closely related taxa (divergences <6%); however, at greater levels of divergence ND2 appeared to evolve much faster, or was less prone to multiple substitutions, than cyt *b*.

Plots of the numbers of transitions in pairwise comparisons against overall percentage sequence divergence showed clear differences between ND2 and cyt *b* (Fig. 2A). Transitions appeared to accumulate at equal rates in the two gene regions for closely related taxa; however, at greater divergences cyt *b* accumulated fewer transitions than ND2 and even showed evidence of multiple substitution at divergences >8%. In contrast, the accumulation of transitions in ND2 as a function of overall diver-

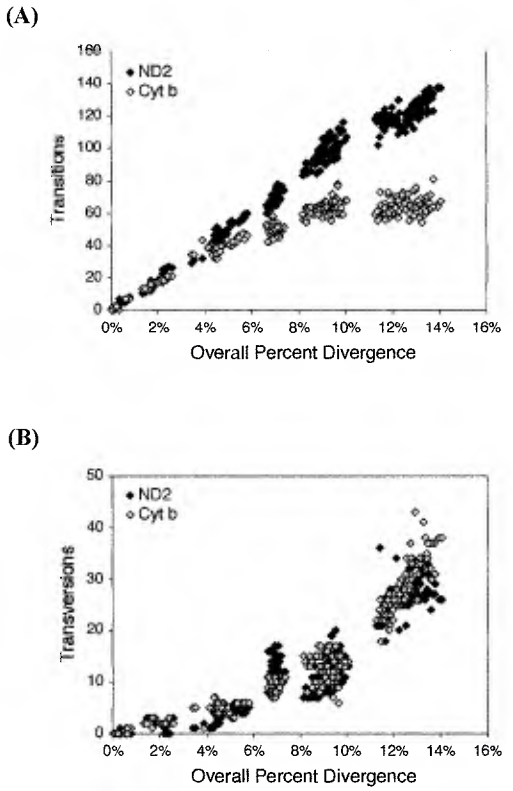

FIG. 2. Plots of (A) transitions and (B) transversions in ND2 and cytochrome *b* against total percentage sequence divergence in pairwise comparisons of all sequences. Transitions in cyt *b* show evidence of multiple substitution at divergences >8%.

gence was linear up to divergences of 14%, with little evidence of such homoplasy. Further analysis revealed that this difference was mostly due to multiple substitutions at third codon positions in cyt *b*. Transversions, in contrast, appeared to accumulate steadily in both ND2 and cyt *b* at approximately the same rate (Fig. 2B). Reconstructed transitions and transversions over the combined unweighted tree also showed differences in the pattern of base substitutions in the two gene regions, consistent with the results from pairwise comparisons. ND2 exhibited a greater reconstructed transition:transversion ratio than did cyt *b* (5.0 vs. 3.1). Altogether, these results suggest that the higher rate of evolution in ND2 can be mostly explained by differences in accumulation of transitions, mostly at third positions, in the two genes. Furthermore, those differences appear to result from multiple substitutions in cyt *b* at higher taxonomic divergences (i.e. comparisons above the species level in our data set). Similar patterns have been found in other studies using these two gene regions (e.g. Hackett 1996, Johnson and Lanyon 1999, Omland et al. 1999, Johnson and Clayton 2000, Klicka et al. 2000, but for an exception see Johnson and Sorenson 1998).

Relationships among the oropendolas.—Parsimony analyses of the combined data set revealed two monophyletic groups of oropendolas (Fig. 3): a clade consisting of *Ocyalus latirostris* and *Psarocolius oseryi* (the *Ocyalus* group) and a clade including all other oropendola taxa (the *Psarocolius* group). These groups were insensitive to weighting scheme. Trees resulting from all the different weightings of transversions over transitions on the combined data set, even 15:1, were identical in the topology of oropendola taxa. The 50% bootstrap tree for each weighting option resolved the same 20 ingroup nodes, 18 (90%) of which received at least 95% bootstrap support. Twelve nodes (60%) received 100% bootstrap support regardless of weighting scheme. Although the consensus tree indicates that the relationship between the two oropendola clades is unresolved (Fig. 3), it should be noted that no analysis supported a monophyletic assemblage of oropendolas.

Decay index values on the unweighted tree (Fig. 3) provide a similar conclusion about the robustness of this topology. All but four nodes were stable when trees 5 steps longer than the shortest tree were retained, and only three additional nodes changed when trees 10 steps longer were retained. All of these less-stable nodes are intraspecific comparisons. Likewise, a jackknife manipulation of taxa indicated that all nodes were consistent with 100% of pseudoreplicates. Oropendola relationships were also relatively unaffected by phylogenetic method used. The maximum-likelihood analyses (Fig. 4) and neighbor-joining analysis all produced trees with oropendola topologies identical to that of the parsimony tree.

Lower bootstrap values were found at just three locations on the variously weighted combined-data tree (nodes A, B, and C in Fig. 3). Two of those nodes are intraspecific comparisons. The sister relationship of *Psarocolius decumanus melanterus* and the *P. d. maculosus* rep-

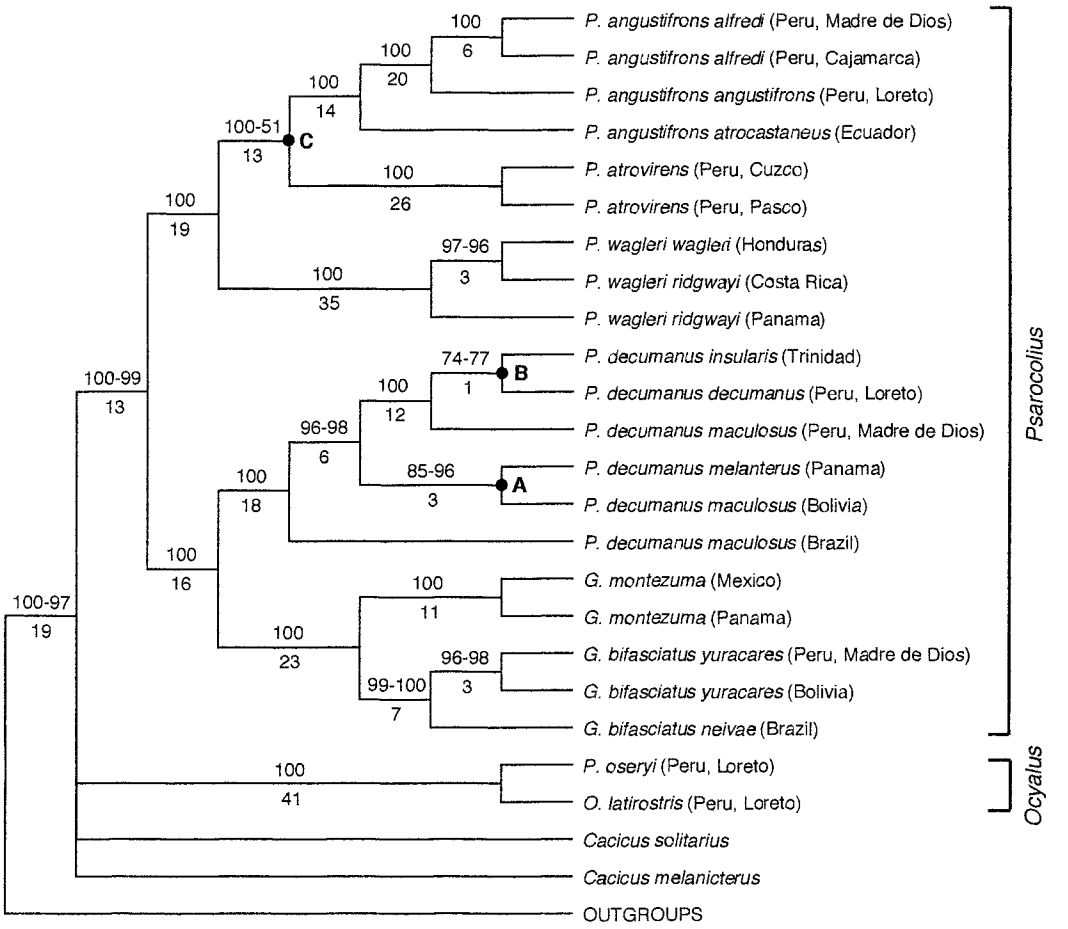

FIG. 3. Consensus of all trees constructed from cyt-*b* and ND2 sequences using the following methods of analysis: maximum parsimony with transversion:transition weightings of 1:1, 2:1, 4:1, 6:1, 8:1, 10:1, and 15:1; maximum likelihood using two models of base substitution; and neighbor-joining. All methods supported an identical ingroup topology consisting of two oropendola clades: the *Psarocolius* group and the *Ocyalus* group. Bootstrap values (above branches) show the range, if any, in nodal support when transversion weighting was increased from 1:1 to 15:1 in parsimony analyses. Decay index values (below branches) pertain to the unweighted parsimony tree (l = 1419, CI = 0.67, RI = 0.81). Jackknife proportions on the unweighted tree were 100% for all nodes shown. Nodes A, B, and C represent relationships that received <95% bootstrap support under some weighting schemes.

resentative from Bolivia (node A) received <95% support with transversion weightings of 1:1 and 2:1 (85 and 91%, respectively), but was supported at or >95% level with higher weightings. The placement of *P. d. insularis* and *P. d. decumanus* as sister taxa (node B) received ~75% bootstrap support in every weighting scheme. Support for the position of *P. atrovirens* as sister to the *P. angustifrons* species group (node C) steadily decreased with increased weighting of transversions over transitions, from 100% in the unweighted tree to 51% with a transversion weighting of 15:1, which suggests that those two types of character transformation support different topologies at this location. (Additional analyses with transversion weightings higher than 15:1 revealed that this alternative topology grouped *P. atrovirens* with *P. wagleri*.) Further investigation (see below) revealed that this inconsistency was restricted to the cyt *b* data set.

In the parsimony analyses of the two genes separately, the ingroup topologies of the variously-weighted ND2 and cyt *b* trees were com-

pletely compatible with each other and with the combined tree. No significant incongruence was found between the phylogenies in a partition homogeneity test ($P = 0.87$) and, like the combined tree, both were surprisingly robust over the range of weighting schemes, with >95% bootstrap support for most nodes (at least 12 ingroup nodes in each gene tree with each weighting option). Under each weighting scheme, roughly half of the nodes in the separate trees showed an increase in bootstrap support in a combined analysis.

The patterns of change in each gene tree under different manipulations suggests the sources of low bootstrap values in the combined-data tree (nodes A, B, and C in Fig. 3). For ND2, node A was unresolved in the unweighted parsimony tree; however, that polytomy disappeared when transversions were weighted over transitions or when bases at first codon positions were removed from the unweighted analysis. For cyt *b*, bootstrap support for node C decreased as transversion bias increased until, at a weighting of 6:1, this node collapsed and *Psarocolius atrovirens* was resolved as sister to *P. wagleri* rather than to *P. angustifrons*. That shift in topology disappeared when third codon positions were removed from the analyses. Thus, low bootstrap support for nodes A and C in the combined-data tree appeared to result, respectively, from at least one first position transition in ND2 and third position transversion in cyt *b* supporting alternative topologies. Node B was unresolved in the cyt-*b* tree in all weighting schemes, suggesting a lack of phylogenetic signal in this data set rather than a conflict.

Relationships between oropendolas and caciques.—Although the topology of oropendola relationships was nearly invariant across the range of weighting schemes and variety of phylogenetic methods used, the relationships to and among some more distant taxa changed dramatically (Fig. 5). In particular, the positions of the two caciques shifted in relation to the two oropendola clades (shown as components of a polytomy in Fig. 3). The unweighted 50% bootstrap tree from the combined data set placed *Cacicus melanicterus* as sister to the *Ocyalus* group and *C. solitarius* as basal to the entire ingroup (Fig. 5A). However, with higher levels of transversion bias those relationships shifted so that, with weightings of 6:1 or more, *C. solitarius* became sister to the *Psarocolius* group and *C. melanicterus* moved to the basal position (Fig. 5B). An identical tree was produced in additional analyses with weightings of 20:1 and 25:1. That topology was also found in the two maximum-likelihood analyses (compare Figs. 4 and 5B). Both of the topologies in Figure 5A and B, it should be noted, represent a single network that differs only in the placement of the root. In contrast, the neighbor-joining analysis placed both caciques between the two oropendola groups on the tree, so that *C. melanicterus* was sister to the *Psarocolius* group and the *Ocyalus* group was basal (Fig. 5C).

No method of analysis supported monophyly of the oropendolas (Fig. 5D). Constraining the topology to find a monophyletic oropendola group required six additional steps in the unweighted parsimony analysis and seven additional steps in the 6:1 weighted analysis. Similarly, a neighbor-joining tree in which oropendola monophyly was enforced had a higher minimum evolution score than one with no such constraint (0.69165 vs. 0.68960, respectively). In contrast, all analyses strongly supported the monophyly of the oropendolas and caciques together (Figs. 3 and 4). That node was strongly supported by jackknife pseudoreplicates and the decay index and received high bootstrap support (97% or above) in all weighting schemes.

Discussion

The monophyly of oropendolas was not supported in our analyses. No weighting scheme or phylogenetic method, using the cyt *b* and ND2 data sets separately or in combination, produced a topology supporting the monophyly of those taxa in relation to the caciques. Rather, our mtDNA sequence data indicated that the oropendolas are polyphyletic with respect to the caciques and are divided into two distinct monophyletic groups: the *Psarocolius* group and the *Ocyalus* group. Both clades received at least 99% bootstrap support in all weighting schemes. The relationships among the two cacique species and the two oropendola clades were not consistently resolved from this data set; however, all of our analyses supported a single, fully-resolved, well-supported tree describing relationships within the oropendolas (Fig. 3).

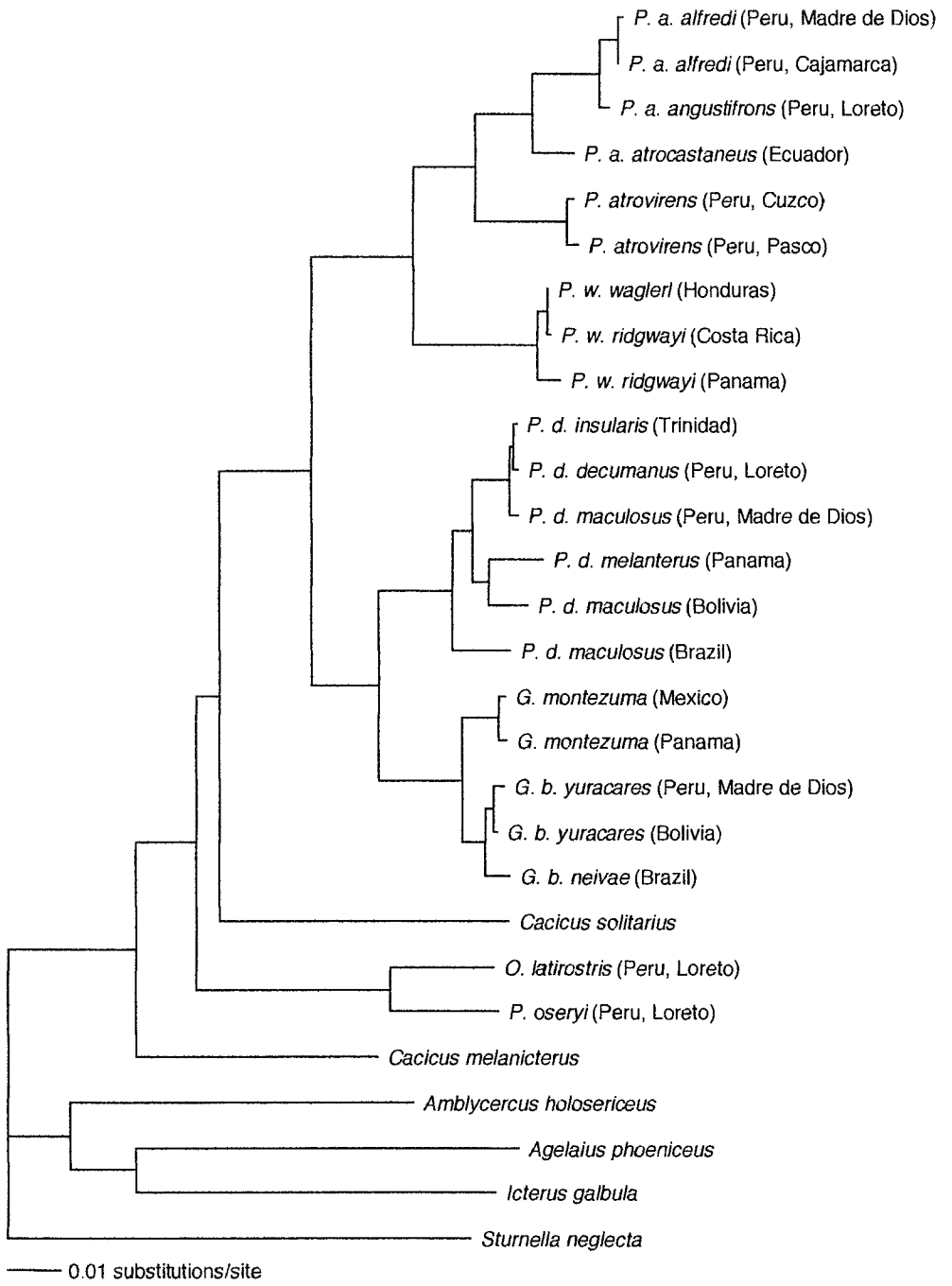

FIG. 4. Maximum-likelihood tree derived using the general time-reversible model of base substitution with invariable sites and gamma distributed rates at variable sites (−lnL = 9255.20; proportion of invariable sites = 0.60; gamma shape parameter = 2.28; rate matrix components: R_{ac} =1.93, R_{ag} = 30.29, R_{at} = 1.27, R_{cg} = 0.22, R_{ct} = 23.08, R_{gt} = 1.00; molecular clock not enforced). Branch lengths are proportional to the number of substitutions. The same topology was found in a maximum-likelihood analysis using the transversion model of sequence evolution (−lnL = 9257.61) and in maximum-parsimony analyses with transversion:transition weightings of 6:1 or greater.

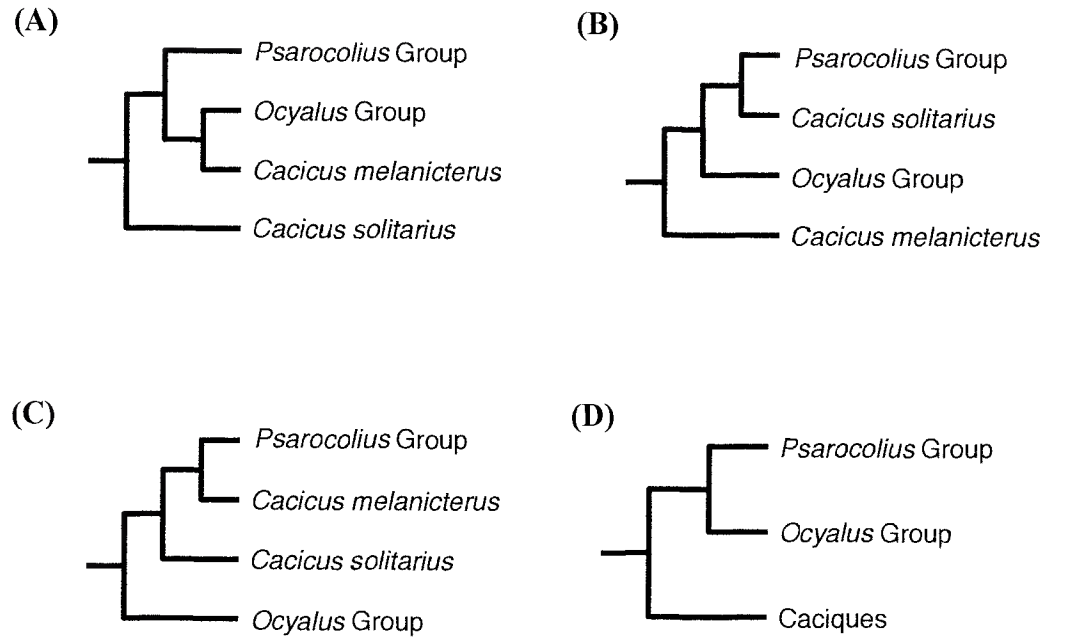

FIG. 5. Hypotheses of oropendola–cacique relationships supported by different methods of analysis: (A) unweighted parsimony tree (l = 1419), (B) maximum likelihood tree (see Fig. 4) and maximum-parsimony tree with transversion:transition weightings of 6:1 or greater (l = 2546 in 6:1 weighted analysis), (C) neighbor-joining tree (ME = 0.6896), and (D) tree supporting monophyly of oropendolas, which was not found in any test.

Our sequence data resolved all 20 nodes in the oropendola tree, with 18 (90%) or more nodes receiving at least 95% bootstrap support in each parsimony weighting scheme. This, it should be noted, was in spite of the fact that our range of transversion weightings extended far beyond those employed in most similar studies (e.g. Barker and Lanyon 2000). Such consistently high resolution and strong nodal support, regardless of the analytical approach used, is unusual in avian molecular systematics and in molecular systematics as a whole. Sequence data are normally characterized by high levels of homoplasy (e.g. Griffiths 1997), which frequently results in polytomies, reduced nodal support, and production of incompatible topologies under different methods of analysis. Indeed, in none of the three previous investigations of blackbird relationships using both ND2 and cyt-*b* sequence data (Johnson and Lanyon 1999, Omland et al. 1999, Klicka et al. 2000) was every ingroup node resolved. Furthermore, even the best-supported tree in each of those studies had comparatively few nodes with bootstrap support >95%: 14 of 38 nodes (37%) for the grackles and allies (Johnson and Lanyon 1999, fig. 4), 22 of 42 nodes (52%) for the orioles (Omland et al. 1999, fig. 6), and 5 of 38 nodes (13%) for the New World nine-primaried oscines (Klicka et al. 2000, fig. 4). Proportionally more nodes in our tree were supported above the 95% bootstrap level than were supported above the 50% level in any of these previous studies.

Unresolved polytomies in a phylogenetic tree are often the result of data sets that are uninformative about a particular period of evolutionary history. Such polytomies are to be expected, however, if new lineages emerged over a relatively short period of time (i.e. during an evolutionary radiation). The absence of polytomies or unstable nodes in the oropendola tree rules out the possibility of such rapid radiations during oropendola evolution. The stability of our oropendola topology also implies a remarkable congruence in the phylogenetic signal of characters within our sequence data. At these levels of divergence (mean ± SE percentage sequence divergence between species: 5.97% ± 0.11, within species: 1.29% ± 0.17),

both genes exhibit similar linear accumulations of transitions and transversions (Fig. 2) and appear to evolve at approximately the same rate (Fig. 1). Investigations of the separate data sets revealed only one notable inconsistency in each. Combining the data sets improved resolution and support for the tree, most likely because this addition swamped any effects those few conflicting characters had on topology.

Despite clear resolution at the species level and below, our sequence data proved much less effective for resolving relationships at higher levels of divergence. Almost every phylogenetic method used, including unweighted parsimony, maximum likelihood, and neighbor-joining (Fig. 5A–C), supported a different hypothesis of relationship for the oropendola clades and the caciques. Average (±SE) percentage sequence divergence values between the caciques and oropendolas (8.94% ± 0.06), the two oropendola clades (9.38% ± 0.06), and the two caciques (9.65%) are all above the level at which transitions in cyt *b* show evidence of multiple substitution (Fig. 2A). Consequently, as found in other recent investigations of the Icterini using cyt *b* (Johnson and Lanyon 1999, Lanyon and Omland 1999, Omland et al. 1999), the lower resolution at deep (basal) nodes might be explained by homoplasy in those characters in comparisons above the species level. If transition saturation is indeed the main source of homoplasy in our data set, we would expect to see increased support for one particular topology when transversions are given greater relative weighting. In fact, heuristic searches with transversion biases of 6:1 or greater produced identical, fully resolved trees, and bootstrap support for deep nodes tended to increase with increased transversion bias. Moreover, this weighted parsimony tree was identical to the topology generated using maximum-likelihood analysis (Figs. 4 and 5B). Based on that evidence, we consider this topology to be our best estimate of relationships among those taxa.

Our results corroborate the study of Lanyon and Omland (1999) in strongly supporting the monophyly of an oropendola and cacique group. Our findings also agree in supporting the sister relationship of *Psarocolius angustifrons* and *P. atrovirens*. However, this earlier study, based on cyt-*b* sequence data, failed to resolve more than one node among the four oropendola species representatives included (Lanyon and Omland 1999). In the present study, which includes between two and six representatives of each of these species, the cyt-*b* data alone resolved all of these relationships with high bootstrap support, and the addition of another mitochondrial gene increased nodal support even more. Thus, the comparatively high resolution and support of our tree confirms the effectiveness of two techniques known to increase tree reliability: dense taxon sampling (Hillis 1996, Graybeal 1998, Omland et al. 1999) and the inclusion of additional sequence data (Bull et al. 1993, Hackett 1996, Johnson and Lanyon 1999).

Our results are also consistent with Freeman and Zink's (1995) mitochondrial restriction-site study in supporting the polyphyly of oropendolas. Similar to our findings, they placed *P. oseryi* and *Ocyalus latirostris* as sister species (our *Ocyalus* group), separate from the other oropendolas. In contrast to our findings, however, their best-supported tree (fig. 1b in Freeman and Zink 1995) grouped these two oropendolas and the two cacique species used in our study, *Cacicus melanicterus* and *C. solitarius*, in their own clade, reciprocally monophyletic with the other oropendolas and caciques. None of our results support that arrangement (Fig. 5A–C).

The monophyly of recognized oropendola species was strongly supported by our analyses. The genera *Psarocolius* (excluding *P. oseryi*) and *Gymnostinops* were also shown to be monophyletic. Below the species level, however, phylogenetic relationships suggested by our mitochondrial sequence data do not correspond well to recognized subspecies limits. For example, *P. decumanus maculosus*, the southernmost race of the Crested Oropendola (*P. decumanus*) (Ridgely and Tudor 1989), was found to be polyphyletic in our results (Figs. 3 and 4). Representatives of that subspecies from three localities were not resolved as each others' closest relatives. The *P. d. maculosus* representative from Brazil was relatively distant from other members of the species; however, the representative from Madre de Dios, Peru, was sister to *P. d. decumanus* and *P. d. insularis*, from Loreto, Peru, and Trinidad, respectively, and the representative from Bolivia was sister to *P. d. melanterus*, from Panama. This last relationship is intriguing because those birds were collected

from opposite edges of the species' range, and it suggests that individuals from locations along the Andes (Panama and Bolivia) are more closely related to each other than to geographically closer lowland forms. An alternative, and more likely, explanation for those patterns, however, is that widespread mitochondrial polymorphism exists in this species, which was sampled by only a few individuals in this study. Such lack of mtDNA support for recognized subspecies divisions is not uncommon (e.g. Ball and Avise 1992, Zink et al. 2000).

Subspecies relationships within the Russet-backed Oropendola complex (*Psarocolius angustifrons*) derived from our data set are consistent with the subspecies limits proposed by Blake (1968) for that species, but not with those of Sibley and Monroe (1990). Blake (1968) divides the species into seven subspecies, three of which were included in our study. Sibley and Monroe (1990), however, divide that species into only two subspecies, *P. a. angustifrons* and *P. a. alfredi*, with representatives from Blake's (1968) *P. a. atrocastaneus* included in the latter taxon. In our phylogeny, Blake's (1968) *P. a. alfredi* was found to be monophyletic and was sister to the nominate form *P. a. angustifrons*, with which it is suspected to hybridize in some parts of its range (Jaramillo and Burke 1999). We resolved the third subspecies, *P. a. atrocastaneus*, as sister to the other two (Figs. 3 and 4). Bootstrap support for these relationships was 100% in all weighting schemes. Thus, our results do not corroborate the subspecies division proposed by Sibley and Monroe (1990) because *P. a. alfredi* and *P. a. atrocastaneus* were not resolved as sister taxa in our study. Nor do our results support the taxonomic split proposed by other authors, who also suggest a sister relationship between those subspecies (e.g. Ridgely and Tudor 1989). Rather, as Jaramillo and Burke (1999) tentatively suggest, the main phylogenetic break in this species might occur between highland forms (including *P. a. atrocastaneus*) and birds of lower elevations (*P. a. angustifrons* and *alfredi*). Indeed, the placement of the highland *P. atrovirens* as the sister to this species complex and the identification of *P. a. atrocastaneus* as sister to the remaining *P. angustifrons* subspecies suggests that the common ancestor was a highland form. That hypothesis can only be examined more rigorously with more complete sampling of the species.

Perhaps our most surprising finding was the sister relationship of *Psarocolius oseryi* and *Ocyalus latirostris*. Both differ substantially from other oropendolas in certain morphological and behavioral characteristics (Koepcke 1972, Leak and Robinson 1989, Ridgely and Tudor 1989, Jaramillo and Burke 1999); however, they also differ from each other in general appearance. The node uniting those two species received 100% bootstrap support in all weightings and the highest decay index value in our analysis (41; Fig. 3). Preliminary analyses with mitochondrial sequence data from cacique taxa not included in the present study (J. J. Price and S. M. Lanyon unpubl. data) suggest that these oropendolas have closer affinities to certain caciques than to other species of oropendola. That possibility is corroborated by the few reported observations of these little-known taxa in the field. Leak and Robinson (1989) describe the social behavior of *P. oseryi*, particularly the breeding system, as more similar to that of the Yellow-rumped Cacique (*Cacicus cela*) than that of other oropendolas. Similarly, Ridgely and Tudor (1989) describe the undulating flight pattern of this species as "rather caciquelike" and different from that of most *Psarocolius* species. The even less well-known oropendola, *O. latirostris*, is similar enough to caciques in overall appearance that it has been suggested as an intermediate between the two groups (Ridgely and Tudor 1989, Jaramillo and Burke 1999). According to Ridgely and Tudor (1989), it is more likely to be mistaken for a cacique than an oropendola in the field. The vocalizations of both taxa are unlike the stereotyped song of most *Psarocolius* and share some characteristics with the variable display songs of certain caciques (Ridgely and Tudor 1989, J. J. Price and S. M. Lanyon unpubl. data). Koepcke (1972) observed that both species construct nests similar to those of some caciques and, curiously, they often nest together in mixed-species colonies.

Both *Psarocolius oseryi* and *Ocyalus latirostris* have been alternately included in and excluded from the genus *Psarocolius* (*P. oseryi* was formerly classified in the monotypic genus *Clypicterus* [e.g. Hellmayr 1937, Beecher 1951]). Our mtDNA data, as well as a variety of corroborating observations, support the exclusion of both taxa from the genus. However, although our analysis reveals a sister relationship between these species, we have yet to obtain

strong evidence indicating which taxa are their closest allies. Until a study of cacique phylogeny can be completed, we consider it premature at this time to propose a change in nomenclature. A more detailed analysis of the oropendola–cacique clade, including mitochondrial sequence data from nearly every recognized cacique taxon, is currently underway (J. J. Price and S. M. Lanyon unpubl. data). The results of that study will further clarify the positions of these taxa within the clade and will lead to a revised classification.

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APPENDIX.

Species	Subspecies	Museum[a]	Catalogue number	Locality
Psarocolius oseryi	(Monotypic)	LSUMZ	120394	Peru, Loreto
Psarocolius decumanus	*decumanus*	LSUMZ	B-27542	Peru, Loreto
Psarocolius decumanus	*insularis*	STRI	TR-PDE1	Trinidad
Psarocolius decumanus	*melanterus*	LSUMZ	164425	Panama, Colón
Psarocolius decumanus	*maculosus*	FMNH	334605	Bolivia, Santa Cruz
Psarocolius decumanus	*maculosus*	FMNH	324065	Peru, Madre de Dios
Psarocolius decumanus	*maculosus*	USNM	B06848	Brazil, Para
Psarocolius atrovirens	(Monotypic)	FMNH	324106	Peru, Cuzco
Psarocolius atrovirens	(Monotypic)	LSUMZ	129462	Peru, Pasco
Psarocolius angustifrons	*alfredi*	FMNH	324068	Peru, Madre de Dios
Psarocolius angustifrons	*alfredi*	LSUMZ	B-32967	Peru, Cajamarca
Psarocolius angustifrons	*angustifrons*	LSUMZ	120397	Peru, Loreto
Psarocolius angustifrons	*atrocastaneus*	LSUMZ	B-7776	Ecuador
Psarocolius wagleri	*wagleri*	STRI	HAPWA-HA29	Honduras
Psarocolius wagleri	*ridgwayi*	LSUMZ	B-27280	Costa Rica, Cartago
Psarocolius wagleri	*ridgwayi*	LSUMZ	B-26395	Panama, Panamá
Gymnostinops montezuma	(Monotypic)	LSUMZ	B-18096	Mexico
Gymnostinops montezuma	(Monotypic)	LSUMZ	164424	Panama, Colón
Gymnostinops bifasciatus	*yuracares*	FMNH	324076	Peru, Madre de Dios
Gymnostinops bifasciatus	*yuracares*	LSUMZ	153616	Bolivia, Santa Cruz
Gymnostinops bifasciatus	*neivae*	USNM	B06889	Brazil, Para
Ocyalus latirostris	(Monotypic)	ANSP	177928	Peru, Loreto
Cacicus melanicterus	(Monotypic)	UWBM	52204	Mexico, Oaxaca
Cacicus solitarius	(Monotypic)	FMNH	324091	Peru, Madre de Dios
Amblycercus holosericeus	*australis*	LSUMZ	98900	Peru, Puno
Icterus galbula	*bullockii*	FMNH	342938	USA, California
Agelaius phoeniceus	*phoeniceus*	FMNH	341893	USA, Louisiana
Sturnella neglecta	(Monotypic)	FMNH	330039	USA, California

[a] ANSP = Academy of Natural Sciences Philadelphia; FMNH = Field Museum of Natural History; LSUMZ = Louisiana State University Museum of Natural Science; USNM = National Museum of Natural History; STRI = Smithsonian Tropical Research Institute; UWBM = University of Washington Burke Museum.