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SONG AND MOLECULAR DATA IDENTIFY CONGRUENT BUT NOVEL AFFINITIES OF THE GREEN OROPENDOLA (*PSAROCOLIUS VIRIDIS*)

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Abstract.—The accuracy with which avian song features indicate phylogenetic relationships has rarely been investigated. In a previous study of vocal evolution in oropendolas (Price and Lanyon 2002a), we estimated the phylogenetic position of the Green Oropendola (*Psarocolius viridis*) using characters derived from oropendolas' elaborate courtship songs. Here, we test that estimate using mitochondrial DNA sequence data. The position of the Green Oropendola determined from molecular data is remarkably close to that based solely on song characters; both data sets support a close relationship between *P. viridis* and members of the genus *Gymnostinops*. Numerous morphological differences yet low genetic divergence among the species in question suggest that rapid diversification has occurred in the group. The fact that this position has not been proposed previously for *P. viridis* underscores the effectiveness of song characters for phylogenetic reconstruction. *Received 27 October 2002, accepted 23 October 2003.*

Resumen.—Existen pocos estudios sobre la exactitud con que las características del canto de las aves indican las relaciones filogenéticas. En un estudio previo sobre la evolución de las vocalizaciones de las oropéndolas (Price y Lanyon 2002a), estimamos la posición filogenética de *Psarocolius viridis* utilizando caracteres derivados de los elaborados cantos de cortejo de estas aves. En este estudio, ponemos a prueba dicha estimación utilizando secuencias de ADN mitocondrial. La posición filogenética de *P. viridis* determinada a partir de los datos moleculares es extremadamente similar a aquella basada sólo en los caracteres del canto; ambos tipos de datos apoyan una relación cercana entre *P. viridis* y miembros del género *Gymnostinops*. La existencia de numerosas diferencias morfológicas con poca divergencia genética entre las especies en cuestión sugiere que el grupo se diversificó rápidamente. El hecho de que esta posición no haya sido propuesta previamente para *P. viridis* resalta la efectividad de los caracteres del canto para la reconstrucción de filogenias.

Various studies have suggested that avian vocalizations contain information about phylogeny (Lanyon 1969; Payne 1986; Irwin 1988, 1996; Spector 1992; Martens 1996; Miller 1996; McCracken and Sheldon 1997; Mahler and Tubaro 2001; Price and Lanyon 2002a). Despite the accumulated evidence, relatively few investigators have used avian vocal characters to directly estimate phylogenetic relationships among taxa; even fewer have tested the accuracy of those estimates using robust, independently derived character sets such as mitochondrial DNA (mtDNA) sequence data. Use of vocal characters is especially uncommon in studies of oscine songbirds (Lanyon 1969, Payne 1986), whose complex songs are generally presumed to be too variable and evolutionarily labile to be useful in systematic investigations.

The oropendolas (genera *Psarocolius*, *Gymnostinops*, and *Ocyalus* [Sibley and Monroe 1990]) are large, colonial-nesting, tropical songbirds in the New World blackbird family (Icterini). In a previous study of vocal evolution in the group (Price and Lanyon 2002a), we used 29 characters derived from their elaborate courtship songs to estimate phylogenetic affinities of three oropendola taxa for which molecular data were not available (*P. viridis*, *P. angustifrons oleagineus*, and *G. guatimozinus*). Those song characters were based mostly on spectrogram measurements and included a variety of temporal and frequency characteristics as well as presence or absence of various complex acoustic patterns. Molecular relationships have been determined for most other oropendolas (Price and Lanyon 2002b), and the song characters we used exhibit a notable lack of homoplasy when mapped onto

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that phylogeny (Price and Lanyon 2002a). Such congruence between song and molecular data suggested that oropendola vocal displays might provide reliable characters for estimating historical relationships.

Here, using mtDNA sequence data, we test our previous song-based hypothesis of relationship for the Green Oropendola (*P. viridis*). The taxonomic placement of this species within the oropendolas has been uncertain historically (e.g. Blake 1968, Ridgely and Tudor 1989, Sibley and Monroe 1990, Jaramillo and Burke 1999), and molecular analyses to address that problem have not been previously conducted. Our analysis of song characters (Price and Lanyon 2002a) found that the Green Oropendola is vocally most similar to the Olive Oropendola (*G. bifasciatus*). Several synapomorphic song features suggest a sister relationship between the two species and thus support inclusion of the Green Oropendola in genus *Gymnostinops*. Counter to that hypothesis, the species differ in a variety of morphological traits; accordingly, no author has previously proposed (to our knowledge) such a classification.

By constructing an mtDNA phylogeny that includes Green Oropendola sequence data, we hope to clarify the position of *P. viridis* within the oropendolas and, in so doing, test the effectiveness of song characters in estimating phylogeny.

METHODS

Tissue from a Green Oropendola representative was obtained from the National Museum of Natural History, Smithsonian Institution (USNM 609202, collected 11 November 1994 in Guyana). Methods employed in DNA extraction, amplification, and sequencing, and the list of primers used in our analysis, are detailed in Price and Lanyon (2002b). In total 2,011 base pairs (bp) of mtDNA sequence were obtained from the sample: 920 bp from the cytochrome-*b* gene and 1,091 bp from the ND2 gene. Those sequences have been deposited in GenBank under accession numbers AY117698 and AY117726. Protocols used in obtaining sequence data from *P. viridis* were identical to those used earlier in sequencing other oropendola taxa (Price and Lanyon 2002b), with the difference that we did not begin sequencing the Green Oropendola sample until after analysis of oropendola song characters (described in Price and Lanyon 2002a) was complete.

Sequence data from 22 other oropendolas, representing multiple subspecies and geographic variants, and from 4 outgroup species were obtained from Price and Lanyon (2002b). The concatenated Green Oropendola sequences were aligned with those others using SEQUENCHER sequence analysis software (Gene Code Corporation, Ann Arbor, Michigan). Outgroup taxa were chosen to represent each of the other four major clades within the blackbirds (Lanyon and Omland 1999): *Agelaius phoeniceus* for the grackles and allies, *Icterus galbula* for the orioles, *Sturnella neglecta* for the meadowlarks and allies, and *Amblycercus holosericeus* for the monotypic cup-nesting cacique clade. Genus and species nomenclature throughout this paper follow Sibley and Monroe (1990), the most geographically comprehensive recent checklist.

All phylogenetic analyses were performed in PAUP* (version 4.0; Swofford 2002). An earlier investigation of oropendola phylogeny (Price and Lanyon 2002b) indicated that parsimony-weighting schemes in which transversions were given ≥6× the weighting of transitions provide the best estimate of relationship in the group. Consequently, a transversion-to-transition weighting of 6:1 in a maximum-parsimony analysis was initially used to estimate Green Oropendola relationships. Sensitivity of those relationships to weighting scheme was explored through additional parsimony analyses with transversion-to-transition weightings of 1:1, 3:1, and 9:1. Degree to which the results were dependent on base composition was determined through full heuristic bootstrap analyses (Felsenstein 1985) under each weighting scheme with 10,000 replicates. Nodal support was also assessed by calculating decay index (Bremer 1994, Eriksson 1999) on the unweighted tree.

To further test stability of Green Oropendola relationships with respect to tree-building method, a maximum-likelihood analysis of the data set was performed using the transversional model of sequence evolution, with invariant sites and with rates at variable sites following a gamma distribution (TVM + I + G, proportion of invariant sites = 0.5941, gamma distribution shape parameter = 2.1287). That model of sequence evolution was selected from among 56 possibilities using a likelihood-ratio test in MODELTEST (version 3.0; Posada and Crandall 1998).

Topologies resulting from molecular analyses were compared, using a Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999), with that estimated from song characters, to see if the trees differed significantly in how well they fit the molecular data.

RESULTS

All of our assorted analyses of molecular data, including maximum likelihood and maximum parsimony under a range of weighting schemes, resolved the same phylogenetic position for the Green Oropendola as sister to genus *Gymnostinops* (Fig. 1). Bootstrap support for that relationship changed little with different weightings of transversions over transitions (87–88% with weightings of 1:1, 3:1, 6:1, and 9:1), and the decay index showed that the node was stable when trees three steps longer than the shortest tree were retained. Our results thus provide good evidence for placing the Green Oropendola at that position, although support for the node was not nearly as strong as values calculated for other interspecific relationships in oropendolas (Fig. 1).

Figure 2 shows position of the Green Oropendola based on song characters (Price and Lanyon 2002a) compared with that based on mtDNA sequence data. The song data resolved a sister relationship between the Green Oropendola and the Olive Oropendola (*G. bifasciatus*; Fig. 2A), whereas the molecular data placed it as sister to both species in the *Gymnostinops* genus (*G. bifasciatus* and *G. montezuma*; Fig. 2B). Those positions differ only in relation to the Montezuma Oropendola (*G. montezuma*), and that difference in topology was not found to be significant in a Shimodaira-Hasegawa test of the molecular data ($P = 0.18$). Thus, our estimate of phylogeny based on oropendola song characters was not significantly worse than that determined from molecular sequence data.

DISCUSSION

Phylogenetic relationships of the Green Oropendola determined here using mtDNA sequence data are similar to those estimated in a previous study (Price and Lanyon 2002a) using comparisons of song characters. Those aspects of song are highly evolutionarily conservative in oropendolas and, consequently, Price and Lanyon (2002a) predicted that they would provide reliable information about relationships among taxa. Although relationships based on song and molecular data differ slightly (Fig. 2), that difference is not significant. The present study thus confirms our previous prediction surprisingly well.

The few previous studies that have used aspects of oscine song to estimate phylogeny provide mostly positive evidence for their effectiveness as systematic characters. Payne (1986), for example, used four synapomorphic song features to construct a phylogenetic tree for five warblers of the genus *Dendroica*, which differs in only one node from a more recent tree derived from mtDNA sequence data (Lovette and Bermingham 1999). Similar results were found by Irwin (1988), who showed that a vocal repertoire containing a single song type is a derived character state defining the sparrow genus *Zonotrichia*. In contrast, Rice et al. (1999) used mtDNA sequences to show that song structure provides little information about relationships in the Winter Wren (*Troglodytes troglodytes*). However, the conclusions of the latter study were based on mostly subjective assessments of song similarity, and not on vocal characters selected using rigorous methods.

A variety of features in the songs of Green and Olive oropendolas (*P. viridis* and *G. bifasciatus*) supported a close relationship between those species in our analysis of song characters (Fig. 2A; Price and Lanyon 2002a). For instance, both produce relatively high-frequency whistles (>8 kHz) at the beginnings of their songs, have nearly identical frequency ranges (~7.5 kHz), and perform rapid shifts in frequency at similar rates (4–6 shifts per second). In contrast, the Montezuma Oropendola (*G. montezuma*) shares few vocal characteristics with other oropendolas (Price and Lanyon 2002a), including *P. viridis* and *G. bifasciatus*. As a consequence, our phylogenetic analysis of song characters, in which positions of taxa other than the Green Oropendola were constrained to the molecular phylogeny, placed the Green Oropendola closer to the Olive than to the Montezuma oropendola. According to our molecular tree, the Montezuma Oropendola's songs appear to have diverged dramatically from the ancestral *Gymnostinops* song pattern (reflected in Green and Olive oropendolas; Fig. 2), and that rapid vocal evolution has evidently obscured much of the phylogenetic signal in the species' songs.

Green and Olive oropendolas are broadly sympatric across much of their respective geographic ranges (Ridgely and Tudor 1989, Jaramillo and Burke 1999). Despite differences in morphology, including different bill colors, eye colors, and overall body sizes, the two species are sometimes confused in the field because of similarities in plumage color (Jaramillo and Burke 1999). That raises the possibility that vocal similarities reported in Price and Lanyon (2002a) were the result not of shared evolutionary history but rather of song

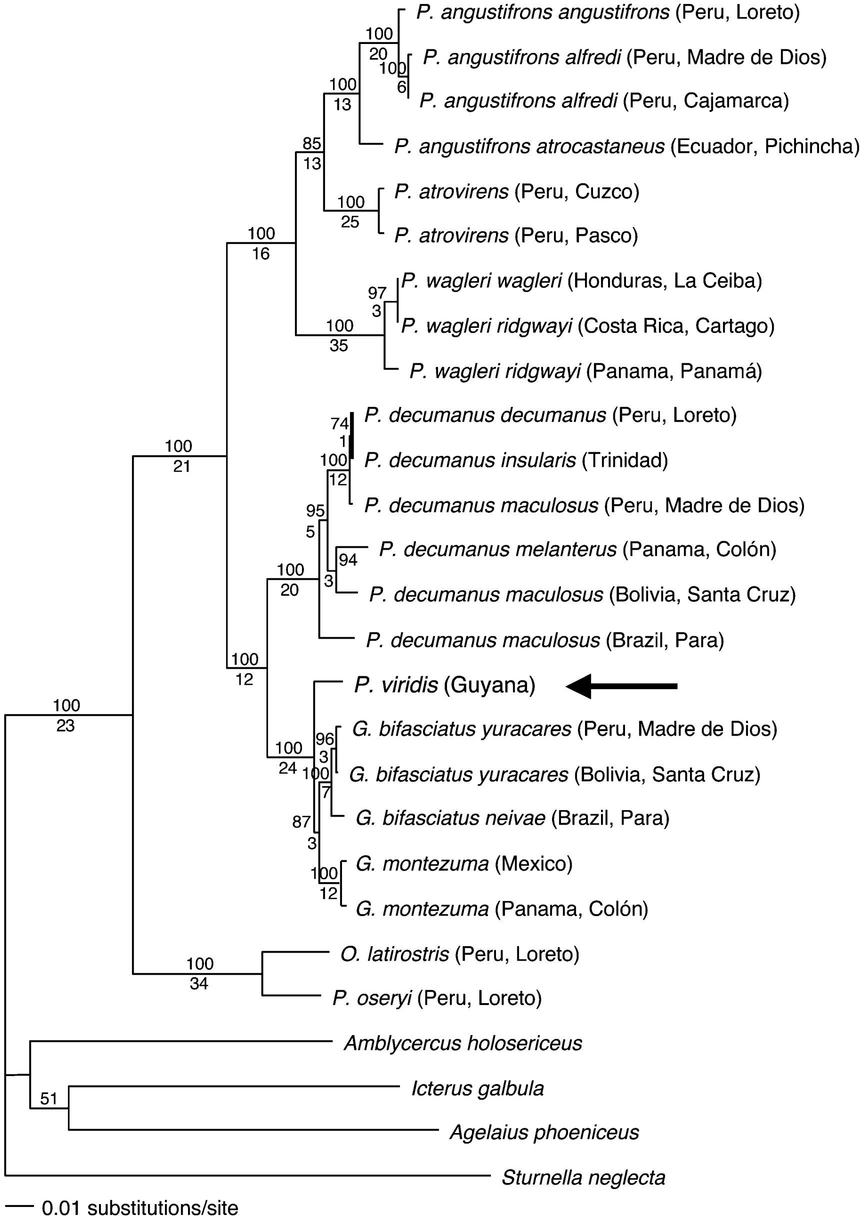

Fig. 1. Maximum-likelihood tree showing phylogenetic position of the Green Oropendola (arrow). Identical topologies were resolved in maximum-parsimony analyses with transversion-to-transition weightings of 1:1, 3:1, 6:1, and 9:1. Numbers above branches indicate percentage of support based on 10,000 bootstrap psuedoreplicates of the 6:1 weighted tree and numbers below branches are decay index values on the unweighted tree (l = 1,238, CI = 0.61, RI = 0.75). Maximum-likelihood analysis followed the transversional model of sequence evolution with invariable sites and with gamma distributed rates at variable sites (–lnL = 8553.90; proportion of invariable sites = 0.61; gamma shape parameter = 2.45; rate matrix components: R_{ac} = 1.95, R_{ag} = 23.81, R_{at} = 1.33, R_{cg} = 0.12, R_{ct} = 23.81, R_{gt} = 1.00; molecular clock not enforced). Branch lengths are proportional to number of base substitutions.

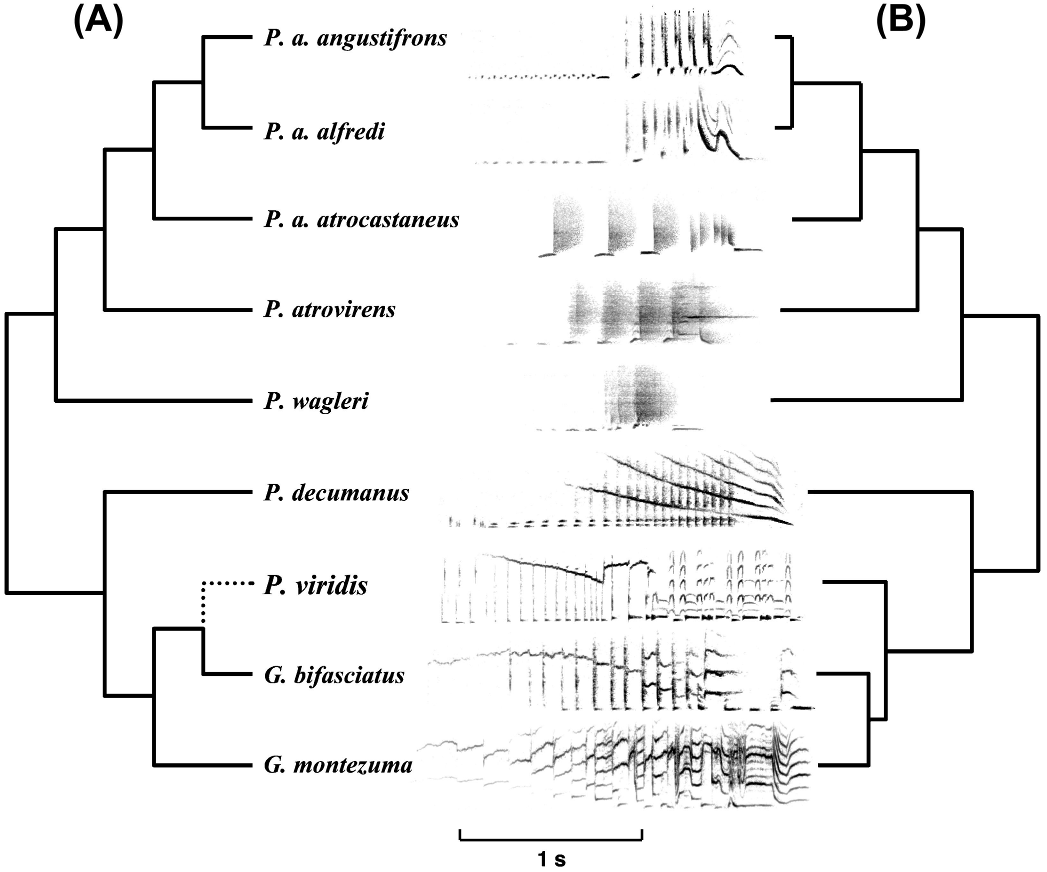

FIG. 2. (A) Phylogenetic relationships of the Green Oropendola (dotted branch), based on heuristic analysis of 29 song characters (l = 56, CI = 0.77, RI = 0.86), with relationships among other taxa (solid branches) constrained to the molecular phylogeny (from Price and Lanyon 2002a). (B) Tree showing molecular relationships of the Green Oropendola based on analysis of mtDNA sequence data (branch lengths reflect the relative number of base substitutions in the unweighted parsimony tree). The two topologies were not significantly different in how well they fit the molecular data (Shimodaira–Hasegawa test: $P = 0.18$). Spectrograms show typical taxon-specific song patterns.

recordists' consistent mistakes in identification (A. Jaramillo pers. comm.). Molecular analysis presented here refutes that idea by showing that the species are indeed closely related. Moreover, there is little chance that DNA samples were collected from misidentified individuals, because representatives of both species were collected in areas of South America in which they are allopatric (Ridgely and Tudor 1989, Jaramillo and Burke 1999, Price and Lanyon 2002b).

Although we consistently resolved the position of the Green Oropendola through a variety of analytic approaches, support for that node was lower than support for other oropendola species relationships (Fig. 1). Areas of reduced support in a phylogenetic tree can indicate periods of evolutionary history when new lineages emerged over relatively short periods of time (i.e. evolutionary radiations). The short internode between the Green Oropendola and the *Gymnostinops* genus (Figs. 1 and 2), as well as relatively low sequence divergence among those taxa (mean ± SE percentage of divergence among Green Oropendola and *Gymnostinops* taxa: 1.85 ± 0.04%; among other oropendola species: 5.97 ± 0.11%), suggests that such a radiation occurred during evolution of the clade. In morphology, the taxa differ substantially from each other and from other oropendolas (Ridgely and Tudor 1989, Jaramillo and Burke 1999), which largely explains their division into separate genera (Sibley and Monroe 1990). Such rapid morphological changes are consistent with rapid changes in song structure that appear to have occurred in some taxa (Price and Lanyon 2002a). Both provide compelling evidence that selection in the group has been especially strong.

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