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POLYPHYLY OF THE BLACKBIRD GENUS *AGELAIUS* AND THE IMPORTANCE OF ASSUMPTIONS OF MONOPHYLY IN COMPARATIVE STUDIES

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Abstract.—A phylogeny for *Agelaius* blackbirds was constructed using sequence data from an 890 base-pair (bp) region of the mitochondrial cytochrome-*b* gene in nine species of *Agelaius* and a single species from all but 1 of the 28 described blackbird genera and subgenera. The genus was found to be polyphyletic with the South American members of *Agelaius* more closely related to other South American blackbird genera. Application of bootstrap and jackknife manipulations supports this conclusion. That this relatively well-known genus is polyphyletic represents a warning to those attempting to construct phylogenies without first demonstrating monophyly of the ingroup. The conclusion that *Agelaius* is polyphyletic necessitates (1) the reinterpretation of previous studies that assumed monophyly and (2) the initiation of a variety of new comparative behavioral and ecological studies suggested by this finding.

Key words.—*Agelaius*, blackbird evolution, comparative approach, cytochrome *b*, DNA sequencing, monophyly, phylogenetic, polymerase chain reaction.

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Few avian genera exist for which hypotheses of evolutionary relationships between the component species are widely accepted. In few genera would such a phylogeny be more useful than for the blackbird genus *Agelaius*. The nine species of *Agelaius* occupy marsh and grassland habitat throughout much of North, Central, and South America and on a few Caribbean Islands. Species within the genus exhibit an impressive range of characteristics: geographic ranges may cover a continent (e.g., *A. phoeniceus*) or be extremely restricted (e.g., *A. xanthophthalmus*); species may nest in colonies (e.g., *A. tricolor*), on territories (e.g., *A. phoeniceus*), or both colonially and territorially (e.g., *A. icterocephalus*); breeding systems include polygyny (e.g., *A. phoeniceus*) and monogamy (e.g., *A. thilius*); species may be sexually dichromatic (e.g., *A. cyanopus*) or monochromatic (e.g., *A. xanthomus*); geographic variation may be extensive (e.g., 23 subspecies of *A. phoeniceus*) or virtually nonexistent (e.g., no subspecies described for *A. humeralis*). The behavioral repertoire, vocalizations, reproductive physiology, breeding behavior, roosting behavior, morphology, migration, biochemistry, cytogenetics, molt, foraging behavior, and general life history of *Agelaius* spp. have been the subject of more than 159 papers in the last 5 yr alone (Zoological Records, volumes 123–127). The red-winged blackbird (*A. phoeniceus*) is certainly the

best-known member of the genus perhaps being the most thoroughly studied passerine in the western hemisphere.

Data from *Agelaius* spp. have played an integral role in the development of many hypotheses in evolutionary biology, including those concerning the evolution of delayed maturation (Rohwer et al. 1980) and of polygyny (Orians 1969; Verner and Willson 1969). However, such hypotheses have been derived in the absence of a hypothesis of phylogenetic relationships within the genus.

Without a basic understanding of evolutionary relationships it is extremely difficult to interpret observations of extant taxa in a historical context. Specifically, without a phylogeny for *A. phoeniceus* and its close relatives, it is impossible to mine the vast knowledge about this assemblage using the comparative approach (Ridley 1983; Felsenstein 1985a; Brooks and McLennan 1991).

Studies of foraging adaptations (Beecher 1951), allelic frequencies (Smith and Zimmerman 1976), cytogenetics (Makino and Baldwin 1954; Hobart et al. 1982), and DNA × DNA hybridization (Sibley and Ahlquist 1990) have produced results relevant to the generic relationships of *Agelaius* but not to intrageneric relationships. The exception is Beecher's (1950) investigation of jaw musculature and plumage patterns in New World

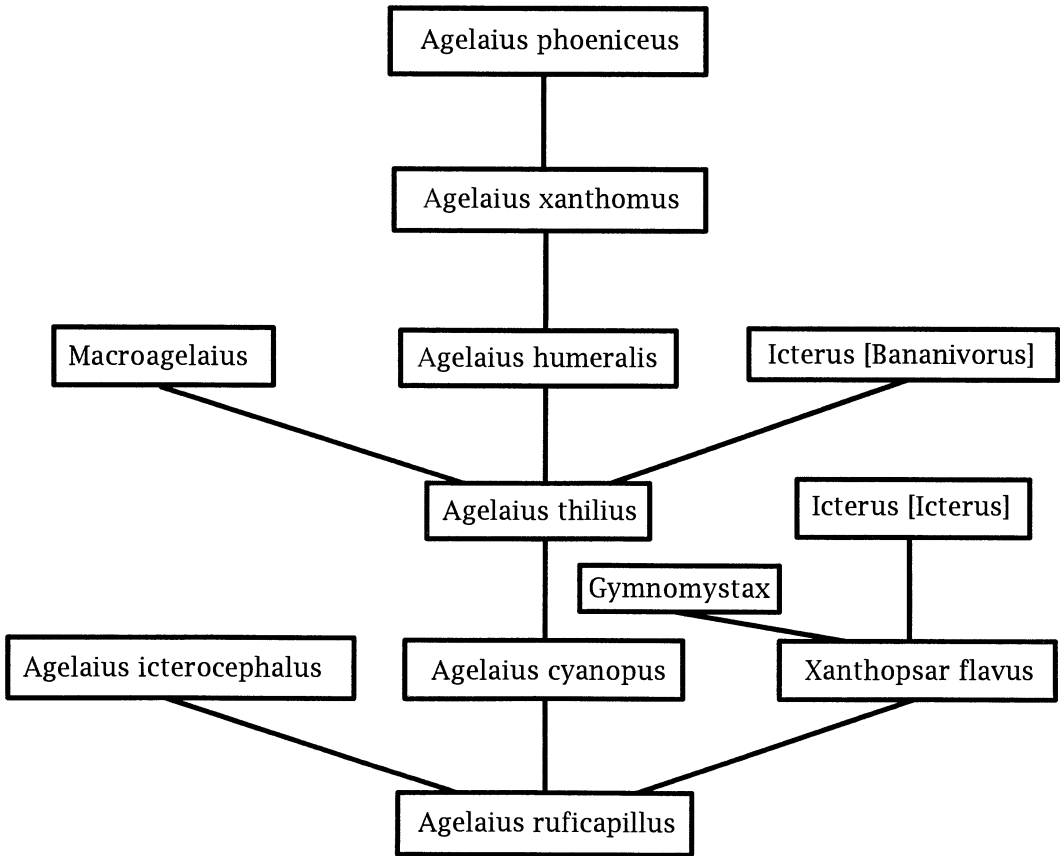


FIG. 1. Evolutionary relationships of *Agelaius* discussed by Beecher (1950) in his analysis of *Icterus* evolution. Placement of extant species at internal nodes is explicit in Beecher's discussion of blackbird evolution.

orioles, in which he hypothesized a rather complicated pattern of evolutionary relationships within *Agelaius*. The phylogeny implied in his paper (fig. 1) suggests that *Agelaius*, as currently recognized, is paraphyletic. In a subsequent paper, Beecher (1951) placed the extant genus *Agelaius* at an internal node from which fully 50% of extant blackbird species are derived *Agelaius* (fig. 2). In this view of blackbird evolution, the similarities among the nine species currently placed in *Agelaius* are best interpreted as the consequence of parallelism not as evidence of close phylogenetic relationship. To maintain the current species of *Agelaius* in a monophyletic assemblage and yet be consistent with Beecher's conclusions, it would be necessary to add 40 species (currently assigned to 10 different genera) to the assemblage. If Beecher is correct, then the conclusions of comparative studies that assume a close relationship between the various *Agelaius*

blackbirds, on the basis on their congeneric status, may need to be reinterpreted.

Although Beecher's analysis constitutes the only direct attempt to reconstruct the evolutionary history of this group, his findings have been criticized (Mayr 1955; Bond 1950) and have not been incorporated in subsequent classifications (e.g., Blake 1968; Morony et al. 1975; American Ornithologists' Union 1983; Sibley and Monroe 1990). Although Beecher's suggestion of paraphyly has not been accepted, considerable uncertainty exists about the limits of the genus. Most other workers have assumed that the species assigned to *Agelaius* are closely related but have suggested that other species be placed in the genus. For example, Bond (1950) and Parkes (1966) have suggested, that *Nesopsar nigerrimus* might actually belong in the genus *Agelaius*. In all, five genera have been proposed as close relatives or possible members of *Agelaius*: *Curaeus*

(Sharpe 1909), *Macroagelaius* (Sclater 1884), *Nesopsar* (Sclater 1884), *Xanthopsar* (Sharpe 1909), and *Xanthocephalus* (Cassin 1866). Thus, neither the limits of nor the relationships within the genus *Agelaius* are now well understood.

The purpose of this study was to produce a phylogeny for the genus *Agelaius*. To ensure that the resulting phylogeny would be useful for a variety of applications in behavioral and morphological evolution, I elected to use biochemical characters as the source of phylogenetic information.

A secondary purpose of this study was to explore the impact of taxon selection on phylogenetic conclusions. The choice of which taxa to include and which to exclude from a phylogenetic study is one of the most important decisions that an investigator must make, and it is often a decision that receives relatively little critical thought. The present investigation of *Agelaius* relationships provides an excellent opportunity to demonstrate the critical nature of this decision. To examine this issue, I conducted three investigations that mimic the kinds of taxon selection decisions made when designing a systematic study. In the first, I included in my presumed ingroup only the nine described species of *Agelaius*. This is the simplest approach in which the current classification is accepted as accurate. Second, I included the nine described species of *Agelaius* as well as those species that at one time were placed within this genus. In this approach to ingroup designation, less confidence is placed on the current classification, and all species that have ever been assigned to the genus are included. In the last investigation, I included the nine species of *Agelaius*, species that at one time were placed within this genus, and at least one member of every other described genus or subgenus of blackbird (except *Hypopyrrhus*). This last approach to designating an ingroup is very conservative. Here confidence is placed on the current classification only at the level of the family.

MATERIALS AND METHODS

Study Taxa

Included in this study were 37 blackbird species including the nine species assigned to *Agelaius*. The remaining 28 taxa include a single species for all but one of the described genera or subgenera of new world blackbirds (*Hypopyrrhus* material was not available for study) [nomenclature follows Blake (1968)]. In addition, four non-

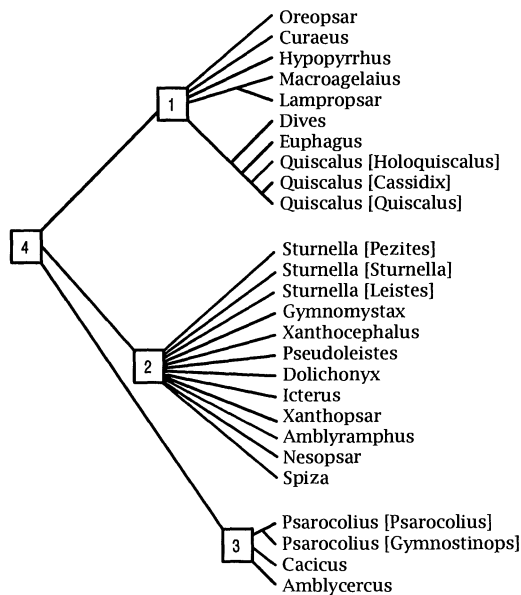


FIG. 2. Evolutionary relationships of *Agelaius* as proposed by Beecher (1951) on the basis of internal morphology, plumage, biogeography, and behavior. Some extant taxa are placed at nodes to be consistent with Beecher's form of argumentation: 1, *Gnorimopsar*; 2, *Agelaius*; 3, *Molothrus aeneus*; and 4, *Molothrus*. Ten extant genera and nine extant *Agelaius* species are shown as descendants of an ancestral *Agelaius*.

blackbird emberizines (*Melospiza melodia*, *Passerina cyanea*, *Saltator coerulescens*, and *Spiza americana*) were included as outgroup taxa. To investigate whether a single individual was sufficient to represent taxa in this study, a second individual of four species (*Agelaius phoeniceus*, *Lamprosar tanagrinus*, *Xanthocephalus xanthocephalus*, and *Dolichonyx oryzivorus*) was examined for a 300 base-pair (bp) segment of the cytochrome-*b* gene. In no instance did intraspecific divergence exceed 1% and, therefore, a single individual specimen was used to represent each taxon. The Appendix lists the specimens used in this study.

Laboratory Methods

A small (50–100 mg) sample of tissue from each taxon was minced with a sterile blade and digested overnight at 37°C in extraction buffer [100 mM tris, pH 8.0, 10 mM Na₂EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, 10 mg/mL dithiothreitol (United States Biochemical), and 0.5 mg/mL proteinase K (Boehringer-Mannheim)]. DNA was extracted twice with equilibrated phenol, once with phenol/chloroform so-

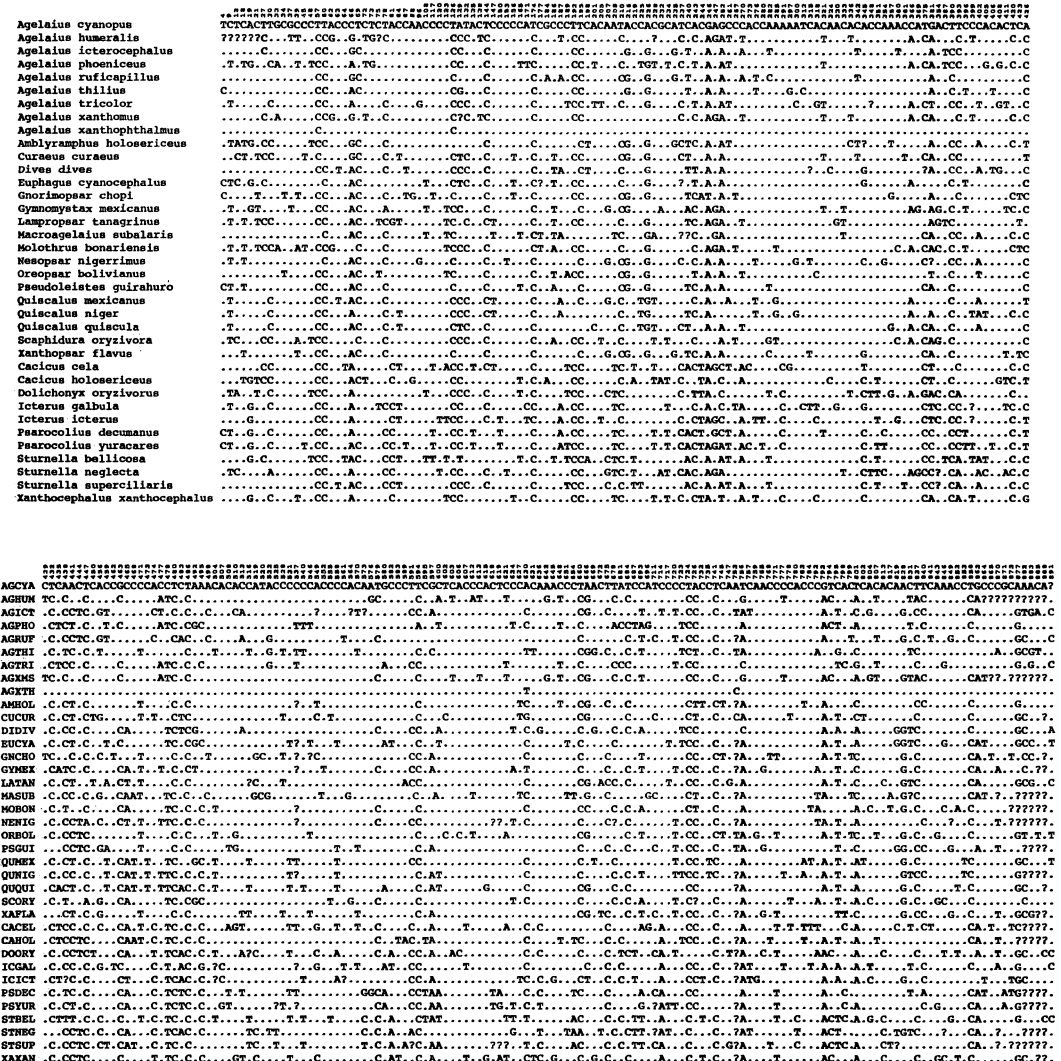


FIG. 3. Shown are 270 potentially informative nucleotide sites derived from an 890 base-pair fragment of the cytochrome-*b* gene. Site numbers refer to the positions within this fragment. Site 1 corresponds to position 15,013 of the chicken mitochondrial genome (Desjardins and Morais 1990). Taxa sharing the same base as *Agelaius cyanopus* are represented by a period to highlight those sites at which taxa differed.

lution (1:1), and twice with chloroform solution (Maniatis et al. 1982). The extracted DNA was ethanol-precipitated, dried, and dissolved in a small volume of distilled water (40–100 μ L). In the initial phase of this study, a 307-bp segment of the mitochondrial cytochrome-*b* was amplified with the so-called "universal" oligonucleotide primers (B1 and B2, fig. 1; Kocher et al. 1989). An additional segment of the cytochrome-*b* gene was amplified with the primers L15042 (5'-ATCTGCATCTACCTACAC-ATCGG-3'; B3) and H15767 (5'-GATGA-

ATGGGTGTTCTACTGGTTG-3'; B4), which targets a 726-bp fragment overlapping that of the fragment produced with B1 and B2. Then, single-stranded amplification products were produced and sequenced with primers B1, B3, B4, and B5 (L15243; 5'-ACCTAGTAGAATGAGCCT-GAGG-3'). L and H refer to light and heavy strands, respectively. Numbers correspond to the location of the 3' end of the primer in the human mtDNA sequence (Anderson et al. 1981). Alternatively, the double-stranded amplification product was freed of remaining nucleotides and

primers, using a glass-powder suspension (GeneClean, Bio101, Inc.) and sequenced directly by the protocol of Thein (1989), modified by the addition of 10% dimethyl sulfoxide to annealing, labeling, and termination reactions. Sequence for an 890-bp region of the cytochrome-*b* gene was obtained. For a complete explanation of polymerase chain reaction (PCR) and sequencing methodology see Lanyon and Hall (1994). The cytochrome-*b* segment data were readily aligned by visual inspection; no insertions or deletions were observed. The character-state distribution for the 270 potentially informative sites is presented in figure 3.

Data Analysis

Initially, the "random-trees option" of PAUP 3.0s (Swofford 1991) was used to determine whether sufficient structure was evident in the data set to warrant further analysis. Ten thousand random trees were generated, and the resultant tree length distribution tested for significant departure from random expectations (Hillis and Huelsenbeck 1992). In all analyses, the results were highly significant ($P < 0.01$) suggesting that further analysis of the data set was warranted. The sequence data set was subjected to a parsimony analysis using PAUP 3.0s (Swofford 1991). Given the many taxa and characters, an exhaustive search of all possible topologies was not possible. Consequently, a heuristic search was employed. Because there are many important assumptions that are made when conducting a heuristic search that may affect the probability of finding the shortest tree(s), I conducted many such searches selecting different combinations of options: the "GENERAL" search, "BRANCH SWAPPING" (tree bisection-reconnection; subtree pruning-regrafting; nearest-neighbor interchanges), and "STEPWISE" (random option with 50 replicates). All characters were considered unordered. These searches were performed first with characters unweighted and then using a step matrix with transversions equivalent to five steps and transitions a single step. All searches resulted in the same tree(s). Because the heuristic search option cannot guarantee finding the most parsimonious topology, I employed a series of data manipulations to examine the strength with which each node was supported by the sequence data.

Three manipulations of varying severity were applied: bootstrapping characters (Felsenstein 1985b), jackknifing taxa (Lanyon 1985), and jackknifing taxa (Lanyon 1985) with characters

reweighted by consistency index (Farris 1969). Bootstrapping identifies the degree to which phylogenetic hypotheses are affected by modifications in the choice of nucleotide sites for analysis. To a large extent, this represents an examination of the number of sites that support each of the various phylogenetic hypotheses. Jackknifing identifies the degree to which phylogenetic hypotheses are affected by modifications in taxon selection. This is a means of estimating the effect of missing taxa on a study. In the final analysis, characters were reweighted as a function of their goodness of fit, as determined by the consistency index (CI), to the shortest trees found in a cladistic analysis of unweighted characters (Farris 1969; Carpenter 1988). These weighted characters were then reanalyzed with a jackknife manipulation of taxa.

Results from these data-set manipulations were nested. That is, nodes stable to a bootstrap analysis (level I stability) were also stable to jackknife analyses of unweighted and of weighted characters. All nodes stable to a jackknife manipulation of unweighted characters (level II stability) were also stable to a jackknife manipulation of weighted characters (level III stability). All nodes stable to these three manipulations were found in the most parsimonious tree for the unmanipulated data set (level IV stability). Use of these manipulations makes possible the identification of each branch by the level of support it receives from the data with level I being strong and level IV relatively weak support.

RESULTS AND DISCUSSION

Sequence Information

Of the 890 sites of the mitochondrial cytochrome-*b* gene sequenced in this study, 270 were potentially informative for a phylogenetic study (i.e., had a minimum of two character states each possessed by a minimum of two taxa). Of these, 48 were codon position #1, 3 were position #2, and 219 were position #3. Transition/transversion ratios and the matrix of pairwise sequence divergences are presented in table 1.

Relationships within Agelaius

The simplest approach for obtaining a phylogeny for *Agelaius* is to assume that the genus, as currently classified, is monophyletic. Parsimony analysis of relationships among the nine species of *Agelaius* using two presumed distant relatives (*Dolichonyx oryzivorus* and *Sturnella*

TABLE 1. Pairwise comparison of cytochrome-b sequence data. Percent nucleotide sequence divergence is presented above the diagonal and the transition/transversion ratio is presented below the diagonal.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 <i>Agelaius cyanopus</i>	0.0	9.5	9.1	10.5	7.7	8.0	8.7	8.8	1.1	8.5	8.8	8.7	8.9	9.4	9.9	9.7
2 <i>Agelaius humeralis</i>	3.4	0.0	9.4	7.8	8.9	10.0	7.4	1.8	8.8	8.1	8.7	8.1	8.3	9.7	9.4	8.6
3 <i>Agelaius icterocephalus</i>	2.7	2.5	0.0	10.2	4.8	6.8	9.0	8.1	8.1	7.2	7.3	7.9	8.3	7.4	8.4	7.8
4 <i>Agelaius phoeniceus</i>	2.7	3.1	2.4	0.0	9.9	10.3	7.0	8.5	9.7	8.7	9.9	8.1	8.0	11.3	10.7	10.3
5 <i>Agelaius ruficapillus</i>	3.2	2.7	4.3	2.5	0.0	6.7	8.2	8.2	6.8	7.4	6.7	6.8	8.2	7.9	8.6	8.4
6 <i>Agelaius thilii</i>	3.7	3.4	2.9	3.0	3.5	0.0	8.7	9.2	7.0	7.8	7.5	7.6	8.1	7.7	7.7	8.1
7 <i>Agelaius tricolor</i>	3.1	3.4	2.3	4.2	2.5	2.7	0.0	7.4	8.0	8.4	8.5	7.1	7.6	10.0	9.0	8.9
8 <i>Agelaius xanthopus</i>	4.9	2.8	2.8	4.2	3.7	4.6	5.4	0.0	8.1	8.4	8.6	7.7	8.1	9.2	9.2	8.4
9 <i>Agelaius xanthophthalmus</i>	1.5	2.8	3.1	2.7	3.0	4.2	2.7	4.0	0.0	7.8	7.8	7.8	8.2	8.4	8.9	8.8
10 <i>Amblyramphus holosericeus</i>	3.7	2.9	2.4	2.5	2.8	3.9	2.7	4.5	3.9	0.0	5.8	7.8	8.5	8.2	9.1	7.7
11 <i>Curaeus curaeus</i>	5.6	3.8	6.0	3.4	6.4	6.3	3.7	5.7	5.3	3.6	0.0	7.7	7.9	8.0	9.2	6.8
12 <i>Dives dives</i>	2.4	2.7	2.1	2.4	2.2	2.7	2.2	3.1	2.5	2.6	3.0	0.0	6.4	8.5	8.2	7.7
13 <i>Euphagus cyanocephalus</i>	3.4	5.2	3.5	4.4	3.5	4.5	5.1	8.9	3.8	4.0	5.9	2.7	0.0	8.8	8.7	7.5
14 <i>Gnomimopsar chopi</i>	4.1	3.0	3.0	3.1	3.6	4.7	3.0	4.3	4.3	4.1	5.4	2.5	4.9	0.0	8.6	8.1
15 <i>Gymnomystax mexicanus</i>	2.9	3.9	2.6	3.3	2.6	3.9	3.0	4.6	2.9	3.0	4.4	2.8	4.8	2.8	0.0	8.0
16 <i>Lamprospyr tanagrinus</i>	4.4	2.9	2.6	3.3	3.6	4.1	3.2	4.5	4.6	3.5	3.6	2.6	4.5	3.7	3.1	0.0
17 <i>Macroagelaius subalaris</i>	2.9	3.2	2.9	3.0	3.5	3.8	3.0	3.8	3.1	3.6	3.7	3.9	3.9	3.6	3.4	3.4
18 <i>Molothrus bonariensis</i>	2.3	1.9	2.0	2.6	1.6	2.0	2.1	2.4	2.2	1.8	2.0	1.9	3.3	2.0	2.2	1.5
19 <i>Nesopsar nigerrimus</i>	3.5	3.3	2.5	3.8	2.8	2.8	4.9	5.2	3.7	4.3	4.5	3.1	8.3	4.8	2.7	4.3
20 <i>Oreopsar bolivianus</i>	3.4	3.1	2.3	2.8	2.7	3.6	2.9	4.3	3.5	3.5	5.4	2.4	3.9	3.3	2.8	3.5
21 <i>Pseudoleistes guttathuro</i>	4.3	3.4	2.7	3.1	4.0	3.9	3.1	5.3	4.1	4.0	6.6	2.4	4.8	4.1	2.5	4.7
22 <i>Quiscalus mexicanus</i>	2.6	4.0	2.8	3.6	3.0	3.4	3.5	5.8	2.6	3.4	4.2	3.5	6.1	3.5	4.2	3.4
23 <i>Quiscalus niger</i>	3.3	3.7	2.8	3.8	3.0	3.5	4.2	5.3	3.4	3.9	5.2	4.2	9.0	3.9	4.4	3.7
24 <i>Quiscalus quiscula</i>	3.2	3.5	3.0	3.7	3.3	3.9	3.8	5.0	3.3	3.2	4.5	3.1	6.0	3.4	5.1	3.1
25 <i>Scaphidura oryzivora</i>	2.4	2.5	2.0	2.6	1.7	2.6	2.2	3.3	2.3	2.1	2.2	2.0	3.5	2.3	2.6	2.3
26 <i>Xanthopsar flavius</i>	6.3	4.6	4.0	3.8	5.8	5.2	4.9	7.7	5.5	4.7	9.3	3.3	6.7	4.7	3.5	5.5
27 <i>Cacicus cela</i>	1.8	1.8	1.8	2.2	2.1	2.1	2.3	2.3	1.7	2.0	2.6	2.2	2.9	1.9	2.3	2.1
28 <i>Cacicus holosericeus</i>	2.3	2.1	2.7	2.3	2.6	2.9	2.8	2.8	2.3	2.2	3.7	2.6	3.8	2.3	2.3	2.7
29 <i>Dolichonyx oryzivorus</i>	1.2	1.3	1.3	1.4	1.3	1.3	1.2	1.5	1.2	1.4	1.5	1.2	1.5	1.3	1.3	1.5
30 <i>Icterus galbula</i>	1.8	1.9	1.8	2.3	2.5	2.4	2.2	2.4	1.8	1.9	2.7	2.0	2.5	2.0	2.3	2.1
31 <i>Icterus icterus</i>	1.6	1.6	1.7	2.0	2.1	2.1	2.0	1.9	1.6	1.6	2.4	1.9	2.6	1.9	1.7	1.9
32 <i>Psarocolius decumanus</i>	2.0	1.6	2.3	2.1	2.6	2.3	2.3	2.2	1.9	2.2	3.0	2.4	2.7	2.1	2.4	2.5
33 <i>Psarocolius yuracares</i>	2.1	2.0	2.3	2.5	2.3	2.5	2.5	2.5	2.1	2.1	2.8	2.5	3.0	2.1	2.4	2.3
34 <i>Sturnella bellicola</i>	1.8	1.7	1.8	1.8	1.9	1.9	2.4	2.0	1.9	1.7	2.2	1.6	2.1	1.8	1.7	1.9
35 <i>Sturnella neglecta</i>	1.5	1.2	1.3	1.4	1.3	1.6	1.4	1.4	1.4	1.6	1.9	1.1	1.6	1.5	1.5	1.4
36 <i>Sturnella superciliaris</i>	1.6	1.5	1.5	1.6	1.5	1.8	1.9	1.8	1.6	1.8	2.0	1.5	2.0	1.6	1.8	1.9
37 <i>Xanthocephalus xanthocephalus</i>	2.0	1.6	2.2	1.9	1.9	2.0	2.0	2.1	2.0	2.0	2.8	1.7	2.7	2.1	2.0	3.0

TABLE 1. Extended.

17	9.2	9.1	8.4	9.0	7.8	8.9	9.4	9.1	8.5	8.9	11.7	10.0	12.1	10.6	10.7	11.1	11.7	12.2	11.6	11.3	36	37
9.7	7.7	8.4	9.4	9.0	9.0	9.7	8.5	8.7	8.3	9.5	10.7	9.7	11.5	10.1	10.5	9.5	10.4	11.7	10.8	10.5	9.8	10.0
11.2	9.1	7.2	5.6	9.1	8.0	8.3	8.5	8.3	8.5	6.4	10.5	9.0	11.3	9.3	9.3	10.7	10.7	10.7	10.9	9.0	10.0	10.4
8.3	8.6	7.1	6.3	5.7	8.1	7.8	7.9	8.2	7.8	8.6	11.5	8.9	11.9	10.3	10.4	10.5	10.9	11.1	11.5	11.1	9.4	10.1
8.9	8.2	7.7	7.3	6.2	8.9	8.2	8.3	8.9	8.9	6.4	11.4	10.2	11.9	10.1	10.5	11.3	11.5	11.3	11.5	10.2	9.5	10.1
10.2	8.0	6.7	8.3	8.0	7.7	7.7	8.1	7.6	8.8	10.6	10.6	8.8	10.4	9.3	9.7	9.8	10.1	11.3	10.1	10.0	10.1	10.1
9.5	7.2	8.1	8.6	8.7	8.0	8.4	8.0	8.4	8.0	9.1	10.6	9.8	11.4	10.1	9.9	10.5	11.1	11.2	10.2	10.3	9.9	9.9
8.5	8.4	7.8	8.1	8.6	8.3	7.8	8.1	11.0	9.2	11.2	11.3	9.3	11.2	10.0	10.2	10.7	10.6	11.4	10.9	10.2	9.6	9.6
8.5	8.3	6.8	8.2	6.9	8.4	8.5	8.1	8.7	7.7	7.2	11.3	9.3	11.2	10.0	10.2	10.7	10.6	10.4	11.5	11.0	9.8	9.8
8.7	8.0	7.8	7.9	7.0	8.8	9.1	7.5	7.1	7.5	7.1	11.9	9.6	11.0	10.5	10.5	11.3	11.1	11.1	11.3	10.4	10.0	10.0
8.0	7.4	6.3	8.1	7.4	6.6	6.5	6.6	6.8	6.8	7.9	10.9	9.1	10.7	9.4	9.6	9.4	9.2	11.1	9.7	8.9	9.6	9.6
8.6	7.4	7.7	8.3	7.4	6.5	6.9	6.4	7.2	8.8	11.3	11.3	9.4	10.8	10.0	10.2	9.9	10.3	10.7	9.8	10.3	9.8	9.8
8.6	9.0	8.2	7.9	7.0	9.8	9.5	9.6	9.4	7.2	10.8	11.6	9.9	12.0	11.0	11.0	10.7	11.0	11.6	11.8	10.3	10.5	10.5
8.7	7.7	7.4	8.5	6.9	8.9	8.7	9.0	7.8	7.7	11.6	11.6	9.4	10.9	11.2	10.6	10.7	11.0	10.9	9.8	10.4	11.0	11.0
8.1	7.1	7.6	8.1	7.2	8.0	8.0	7.5	8.2	7.4	11.2	11.2	9.3	11.2	10.1	10.4	11.2	11.1	11.2	10.1	9.5	10.6	10.6
0.0	8.8	8.1	8.7	7.7	9.4	9.0	8.9	9.3	8.9	11.0	11.0	10.2	12.1	10.8	10.6	10.2	11.0	11.1	10.7	10.4	10.9	10.9
1.9	0.0	7.9	8.8	8.2	7.4	7.6	7.4	4.2	8.7	10.7	10.7	9.8	10.4	9.9	9.8	10.5	10.8	11.3	10.3	10.5	9.7	9.7
3.9	2.3	0.0	7.4	5.7	7.0	7.1	7.3	7.1	6.4	9.9	9.9	8.1	10.3	9.0	9.2	9.6	9.2	10.4	9.0	8.9	9.1	9.1
3.2	1.8	4.3	0.0	6.5	9.0	8.7	8.6	9.0	7.1	11.9	11.9	10.0	12.0	11.1	10.5	12.3	12.0	11.5	11.6	10.6	9.6	9.6
3.2	2.1	3.0	4.2	0.0	8.2	7.6	7.5	7.5	4.0	10.7	10.7	9.1	10.3	10.0	10.3	10.7	10.6	10.6	10.1	9.2	10.0	10.0
4.8	2.6	4.9	3.0	3.2	0.0	3.9	5.2	7.0	8.8	11.0	11.0	9.7	10.8	10.1	10.0	10.4	10.5	11.8	10.7	9.6	10.1	10.1
5.0	2.9	5.7	3.2	3.4	10.3	0.0	4.7	7.7	8.6	10.8	10.8	10.1	10.7	9.5	9.7	10.4	10.8	11.0	10.3	9.7	9.8	9.8
3.8	2.6	5.2	3.0	3.1	4.8	4.9	0.0	7.4	8.2	11.3	11.3	9.5	10.1	10.2	9.6	10.3	10.3	10.4	10.3	9.2	9.4	9.4
2.3	8.0	2.8	2.1	2.1	2.9	3.5	3.1	0.0	8.5	11.1	11.1	9.5	10.4	10.0	10.3	10.5	10.4	10.8	9.5	10.3	9.3	9.3
4.5	2.4	4.4	5.9	7.8	4.5	4.8	4.1	2.8	0.0	11.6	11.6	10.2	11.7	10.3	11.3	11.5	11.1	10.9	10.7	10.0	10.5	10.5
2.3	1.9	3.0	2.4	1.8	2.4	2.5	2.5	2.0	2.6	0.0	8.9	11.6	11.6	9.6	9.7	7.8	8.8	10.5	12.0	10.0	9.3	9.3
2.7	2.0	3.1	2.6	2.3	3.2	3.6	3.6	2.2	3.0	2.9	0.0	10.1	10.1	9.4	8.7	8.6	9.1	9.8	10.4	8.6	8.4	8.4
1.3	1.2	1.2	1.3	1.1	1.4	1.5	1.4	1.2	1.5	1.3	1.3	1.3	0.0	11.6	10.5	10.9	11.3	10.8	10.0	9.4	8.2	8.2
2.4	1.8	2.0	2.5	2.1	2.3	2.3	2.3	1.7	2.3	2.0	2.7	1.4	1.2	0.0	7.6	9.3	9.6	11.6	10.9	10.6	9.5	9.5
1.8	1.7	2.0	2.1	1.9	2.2	2.2	2.1	1.7	2.4	1.9	2.3	2.3	1.4	7.3	0.0	8.8	9.2	10.7	11.1	9.9	8.5	8.5
2.8	1.8	2.7	2.7	2.2	2.8	3.0	2.5	2.1	3.2	3.9	2.8	1.3	1.3	2.4	2.0	0.0	4.7	10.4	10.5	9.5	8.5	8.5
3.0	2.0	2.7	2.5	2.2	3.0	3.3	2.6	2.1	2.9	4.8	2.8	1.3	1.3	2.3	2.0	7.2	0.0	10.2	10.6	9.6	9.1	9.1
1.9	1.5	2.8	1.8	1.9	2.3	2.6	2.0	1.4	2.3	2.3	2.3	1.4	1.4	2.1	1.5	2.3	2.3	10.0	10.0	6.5	9.5	9.5
1.5	1.1	1.5	1.6	1.3	1.7	1.9	1.8	1.1	1.6	2.1	2.1	1.5	1.1	1.4	1.3	1.8	1.8	2.6	0.0	7.7	10.1	10.1
1.9	1.3	2.0	1.8	1.5	1.7	1.9	1.6	1.4	2.1	2.6	2.6	1.3	2.1	1.8	1.5	2.3	2.4	3.7	2.0	0.0	9.4	9.4
2.0	1.5	2.5	1.8	2.0	2.3	2.3	2.3	2.1	1.7	2.4	2.2	1.8	2.2	2.2	1.8	2.5	2.6	2.2	1.9	2.4	2.4	0.0

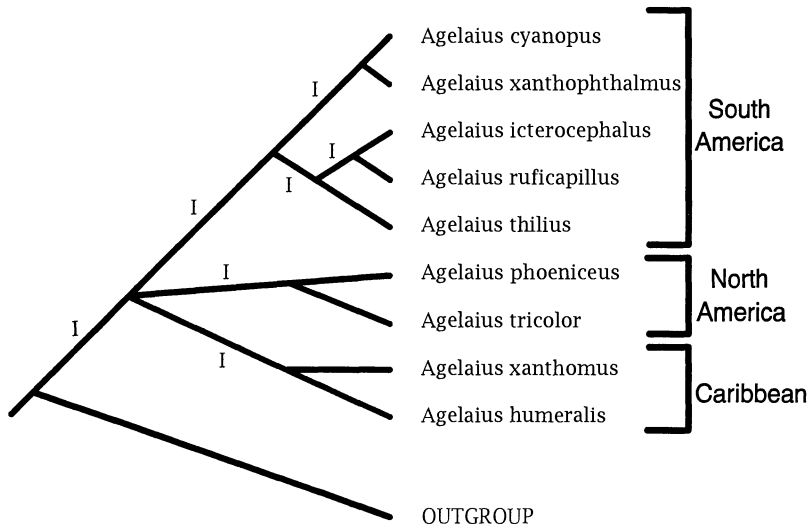


FIG. 4. Strict consensus of the two equally most parsimonious trees (length = 270 steps; CI = 0.622; RI = 0.605) resulting from a cladistic analysis of an 890 base-pair (bp) region (129 potentially informative) of the cytochrome-*b* gene for the nine described species of *Agelaius*. All internal branches were supported by a minimum of 75% of pseudoreplicates in a bootstrap analysis (indicated by "I" to be consistent with subsequent figures). Outgroup taxa used in this analysis were *Sturnella neglecta* and *Dolichonyx oryzivorus*.

neglecta) as outgroup taxa produced two most parsimonious trees (fig. 4). A jackknife manipulation of in-group taxa and a bootstrap analysis (1000 replicates) detected no instability in the nodes identified in figure 4.

This topology indicates that phylogenetic and biogeographic relationships of *Agelaius* are concordant. The two North American species, the two Caribbean species, and the five South American species each form discrete clades.

Relationships within Agelaius and Close Relatives

An alternative approach to investigating the relationships of *Agelaius* blackbirds would be the analysis not only of described species of *Agelaius* but also of those that have been suggested as possible members, or close allies, of the genus. Five genera have been proposed as close relatives or possible members of *Agelaius*: *Curaeus* (Sharpe 1909), *Macroagelaius* (Sclater 1884), *Nesopsar* (Sclater 1884), *Xanthopsar* (Sharpe 1909), and *Xanthocephalus* (Cassin 1866). A parsimony analysis of the DNA data set including these taxa results in a single most parsimonious tree (fig. 5). All nodes were stable to a jackknife manipulation of reweighted characters, seven were stable to a jackknife manipulation, and six to a bootstrap manipulation. (fig. 5).

With the exception of *Xanthocephalus*, analysis of these data with respect to *Sturnella* and *Dolichonyx* places all presumed close relatives within the genus *Agelaius*. Constraining the analysis to produce a topology with a monophyletic clade consisting of the nine *Agelaius* species results in a single tree requiring nine additional evolutionary steps [consistency index (CI) excluding uninformative characters = 0.498]. More than 4500 trees exist that are as short as or shorter than the trees constrained to be monophyletic.

The biogeographic pattern identified in the first analysis is not as clear in this somewhat broader assemblage. A large clade exists containing the five South American *Agelaius*, *Curaeus*, *Macroagelaius*, and *Xanthopsar* (i.e., all South American taxa). The two North American *Agelaius* are identified as sister taxa, but the North American *Xanthocephalus* is the sister taxon to all *Agelaius* and close relatives. The Caribbean *Nesopsar nigerrimus* is the sister taxon of the South American clade. The two Caribbean *Agelaius*, *A. xanthomus* and *A. humeralis*, are sister taxa and together form the sister taxon of the North American *Agelaius*.

Generic Relationships within the Icteridae

In the final analysis, I made the assumption that all close relatives of the described *Agelaius*

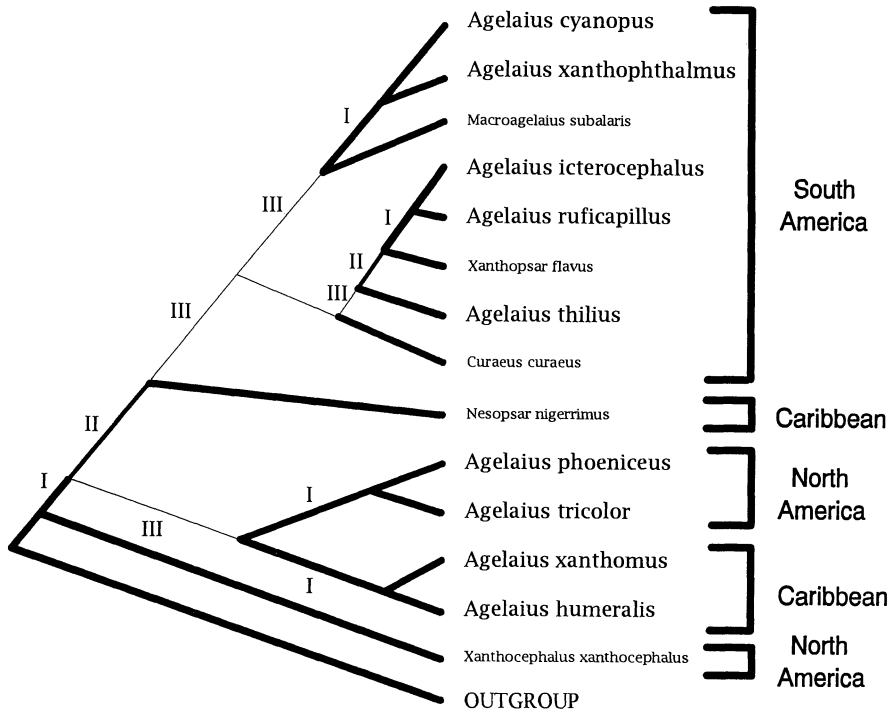


FIG. 5. Single most parsimonious tree (length = 457; CI = 0.508; RI = 0.492) resulting from a cladistic analysis of an 890 base-pair (bp) region (171 potentially informative) of the cytochrome-*b* gene for described species of *Agelaius* and taxa previously assigned to this genus. Roman numerals accompanying internal branches identify their relative stability to various data manipulations (with I most stable). Outgroup taxa used in this analysis were *Sturnella neglecta* and *Dolichonyx oryzivorus*.

species are classified within the Icteridae. I analyzed 37 species representing 28 of 29 genera or subgenera of blackbirds recognized by Blake (1968). Nonicterid out-groups were included in the analysis to root the resultant topology. Root placement varied slightly depending on which out-group taxa or combinations of out-group taxa were included. All possible roots identified by the various outgroup taxa, however, resulted in a monophyletic clade containing the nine described species of *Agelaius* and 15 of the 28 blackbird genera analyzed. This clade was robust to jackknife and bootstrap manipulations of the data set. Parsimony analysis placed *Xanthocephalus xanthocephalus* as a member of the out-group.

To determine relationships within this clade, the sequence data were reexamined. The more distantly related nonicterid taxa were excluded to reduce homoplasy. The remaining 11 blackbird species (*Cacicus cela*, *Cacicus holosericeus*, *D. oryzivorus*, *Icterus galbula*, *I. icterus*, *Psarocolius decumanus*, *Psarocolius yuracares*, *S. ne-*

glecta, *S. bellicosa*, *S. superciliaris*, *X. xanthocephalus*) were used as the out-group. Four equally parsimonious cladograms were identified by PAUP, and the strict consensus of these topologies is presented in figure 6. Of the 24 nodes contained in this topology, nine were stable to a bootstrap manipulation (level I support), four more were stable to a jackknife manipulation (level II support), and five more were stable to a jackknife manipulation of reweighted characters (level III support).

The analyses identified a clade containing the five South American members of the genus and nine additional South American genera. One clade of South American *Agelaius* identified in this study was suggested by Short (1968). In his original description of *A. xanthophthalmus*, he stated that on the basis of "body shape, proportions, color, bill shape, and what little is known of its habits" this all-black species was most closely related to *A. thilius* and *A. cyanopus*. *Agelaius cyanopus* has an all-black adult male plumage and has a song similar to that of *A. xanthoph-*

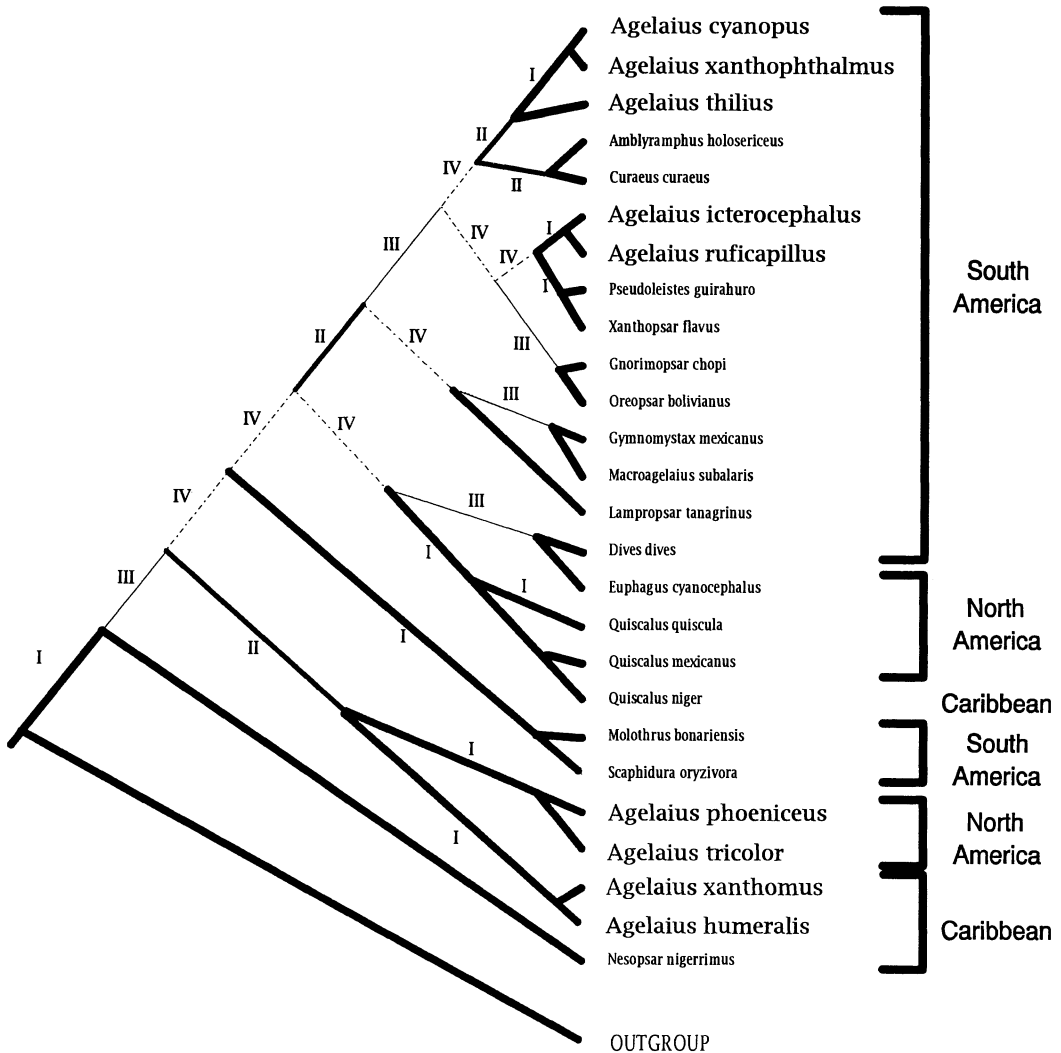


FIG. 6. Strict consensus of the four equally most parsimonious trees (length = 1210; CI = 0.334; RI = 0.430) resulting from a cladistic analysis of an 890 base-pair (bp) region (270 potentially informative characters) of the cytochrome-*b* gene. Roman numerals accompanying internal branches identify their relative stability to various data manipulations with I most stable. Outgroup taxa used in this analysis were *Cacicus cela*, *Cacicus holosericeus*, *Psarocolius decumanus*, *Psarocolius yuracares*, *Dolichonyx oryzivorus*, *Xanthocephalus xanthocephalus*, *Sturnella superciliaris*, *Sturnella bellicosa*, *Sturnella neglecta*, *Icterus galbula*, and *Icterus icterus*.

thalmus (T. Parker pers. comm. 1986). *Agelaius phoeniceus* and *A. tricolor*, the two North American members were identified as sister taxa. The two Caribbean species, *A. xanthomus* and *A. humeralis*, are sister taxa and may represent the sister taxon of the North American *Agelaius* clade. Constraining the parsimony analysis to produce a topology in which the nine *Agelaius* form a monophyletic clade results in two trees 11 steps longer (CI excluding uninformative characters = 0.331). More than 4000 trees exist that are as

short or are shorter than the tree maintaining *Agelaius* as monophyletic. The consensus of these trees retains no information.

Because DNA sequence characters are constrained to one of four possible character states, relatively high levels of homoplasy can result. This is especially problematic when the number of taxa in an analysis is high, as it was in this study. To investigate whether the conclusion that *Agelaius* is polyphyletic might in fact be an incorrect conclusion reached because of high levels

of homoplasy in this very large data set, I conducted an additional modified jackknife analysis. I constructed pseudoreplicate data sets containing two outgroup taxa (*S. neglecta* and *D. oryzivorus*), all nine species of *Agelaius*, and a different blackbird genus or subgenus in each data set. In 15 instances, the additional taxon was placed within *Agelaius* as currently recognized. The placement of a single taxon was ambiguous. Consequently, polyphyly of the genus is strongly indicated by this data set.

CONCLUSIONS

The well-studied New World blackbird genus *Agelaius* was determined, through parsimony analysis of DNA sequence data, to be a polyphyletic assemblage of ecologically similar birds. The hypothesis of *Agelaius* monophyly was consistently rejected in a series of data manipulations. Topologies in which *Agelaius* is monophyletic were substantially less parsimonious than those demonstrating polyphyly. Although the conclusion that *Agelaius* is polyphyletic is consistent with earlier conclusions of Beecher (1951), the actual topology supported by the current study differs substantially from his findings.

It is important to note that the scattering of *Agelaius* species across the topology that is supported by cytochrome *b* is atypical. Monophyly of other genera [*Quiscalus* (fig. 6); *Icterus*, *Sturnella*, and *Psarocolius* (this study but not illustrated)] was strongly supported by these data. As reported elsewhere (Lanyon 1992) and confirmed here (fig. 6), the two genera exhibiting interspecific brood parasitism, *Scaphidura* and *Molothrus*, were identified as sister taxa. That so many hypotheses of monophyly were confirmed supports the conclusion that the mitochondrial cytochrome-*b* gene retains substantial phylogenetic information. Therefore, *Agelaius* polyphyly must be taken seriously and cannot be dismissed simply as an aberrant pattern resulting from analysis of characters lacking phylogenetic information.

In light of the high levels of homoplasy that characterize sequence data it is interesting to note that the topologies produced by analysis of the three data sets (9, 13, and 26 in-group taxa) were largely compatible. That is, the relationships among the nine *Agelaius* species were the same (except for the relationships of *A. thilius* in fig. 6) in all three analyses despite increased levels of homoplasy with increased numbers of taxa. This supports the notion that the phylogenetic

signal is successfully detected by parsimony analysis even when considerable homoplasy is present.

If we are to insist that our classifications contain only monophyletic taxa, then the genus *Agelaius* must either be split into several monophyletic entities or 14 blackbird genera must be lumped with *Agelaius*. If the latter approach were taken, then this one genus, to be named *Amblyramphus* in accordance with priority, would contain 37 species, representing more than one-third of the members of the family. An alternative is the placement of some species of *Agelaius* into other existing genera. However, in my opinion neither action is warranted now. Although these sequence data provide strong evidence that *Agelaius* is polyphyletic, they do not reliably indicate phylogenetic relationships for all currently recognized *Agelaius* species. Figure 7 illustrates the portions of the topology that are strongly supported by the cytochrome-*b* data (level I or II support) and can be used as the foundation for changes in nomenclature or as the basis of tests of evolutionary hypotheses. Consequently, I consider changes in *Agelaius* nomenclature premature now. Such decisions should await an additional data set that will confirm *Agelaius* polyphyly and that will more reliably indicate the affinities of species within the South American clade.

Of what consequence is this finding of polyphyly for comparative studies? Wiley and Wiley (1980) discussed the breeding ecology of the tropical *A. icterocephalus* as compared with that of the north temperate *A. phoeniceus* and *A. tricolor*. By comparing these congeners, the authors hoped to shed light on the behavioral and ecological consequences of life in these disparate environments. The present study indicates that these taxa are not as closely related as assumed. Therefore, the observed differences between these tropical and north temperate taxa could be caused by phylogenetic rather than ecological constraints. The present study indicates that a comparison with the south-temperate *X. flavus* might provide a better opportunity to control for phylogenetic effects.

An investigation of biogeographic patterns provides an explicit example of the problems that can result from making incorrect assumptions of monophyly when reconstructing phylogenies. Based on the results of the first analysis containing only the nine described species of *Agelaius*, we would conclude that the three bio-

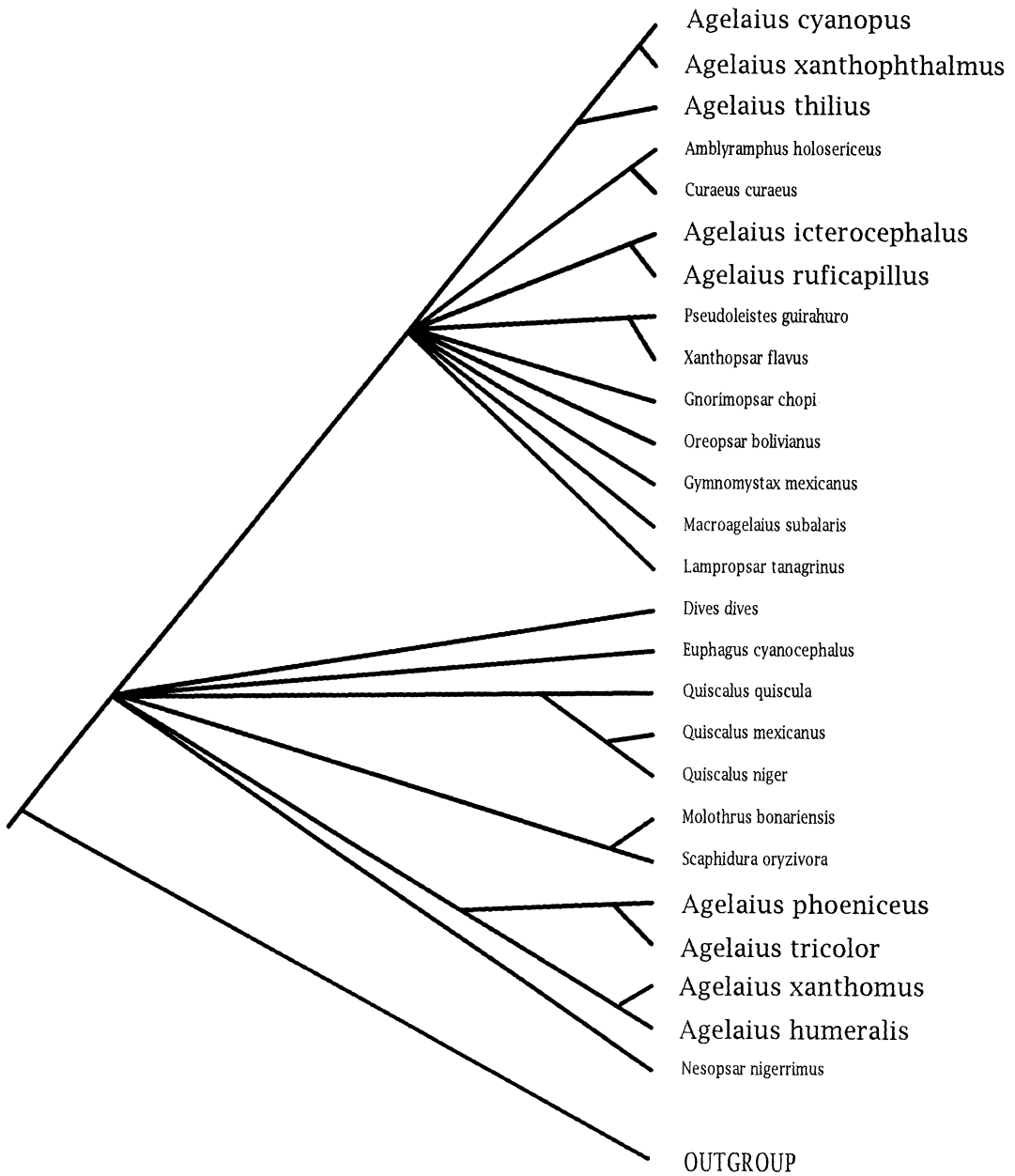


FIG. 7. Partially resolved phylogeny of the New World blackbirds derived from parsimony analysis of an 890 base-pair (bp) region of the mitochondrial cytochrome-*b* gene. Only those nodes found in 75% or more of pseudoreplicates when characters were bootstrapped and/or 100% of pseudoreplicates when taxa were jackknifed have been retained.

geographic regions (North America, South America, and the Caribbean Islands) each contained a monophyletic subset of *Agelaius*. However, as additional taxa are added to the phylogenetic study, our biogeographic interpretation changes significantly. In the most complete anal-

ysis, it appears that the least inclusive monophyletic group containing the nine *Agelaius* species, a group containing 16 additional taxa, has seen a much more complicated biogeographic history. Not one of the three biogeographic regions contains a single monophyletic group of

blackbirds with North America having been colonized a minimum of three times.

Perhaps one of the most surprising results of this study is the distant relationship between *Agelaius* and *Xanthocephalus*. These taxa have long been considered closely related on the basis of behavioral and ecological similarities. Cassin (1866) placed *Xanthocephalus xanthocephalus* within the genus *Agelaius*. Ridgway (1902) recognized the *Agelaii* to contain three genera *Agelaius*, *Nesopsar*, and *Xanthocephalus*, and most classifications place the two genera adjacent to one another (Blake 1968; Morony et al. 1975; Sibley and Monroe 1991). Lack (1968) assumed a close relationship when comparing the ecological similarities of these taxa to the ploceid *Euplectes*. Results of the present study, however, indicate that *X. xanthocephalus* is only very distantly related to the other marsh blackbirds and in fact is more closely related to bobolink, meadowlarks, and so on. Therefore, the similarity in *Agelaius* and *Xanthocephalus* ecology is almost certainly convergent. Given the similarity in breeding habitat of these species and the importance of habitat as a causal factor in several ecological theories, most notably the polygyny threshold hypothesis (Verner and Willson 1966, 1969; Orians 1969), further intensive ecological study of the less well-documented *Xanthocephalus* is clearly warranted.

The relatively distant relationship between *Agelaius* and *Nesopsar* deserves similar attention. Wiley and Cruz (1980) designed a study of evolutionary adaptation based on the explicit assumption that *Nesopsar*'s "closest relatives are blackbirds in the genus *Agelaius*." The authors' intent was to study "two species that share a relatively recent phylogenetic origin but differ in their current environments." The present study suggests that these taxa are not in fact closely related. The forest dwelling *Nesopsar* is identified as the sister taxon of all other "typical" blackbirds, an assemblage that includes grassland, marsh, and forest species. This is an excellent example of the importance of establishing phylogenetic relationships prior to conducting comparative studies in evolutionary biology.

In a general way, the present study demonstrates the dangers inherent in assuming monophyly in the interest of simplifying a study. Confining the investigation to the nine species currently assigned to *Agelaius* (i.e., assumed monophyly) led to a very clean but incorrect answer. Even inclusion of all presumed close rel-

atives failed to produce a reasonable understanding of the evolution of these species. The fundamental importance of assumptions concerning monophyly must be acknowledged by evolutionary biologists employing the comparative approach. If the monophyly of a well-studied genus such as *Agelaius* cannot be trusted then all taxonomic assemblages must be viewed cautiously.

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APPENDIX

List of Voucher Specimens

Field Museum of Natural History (FMNH): *Agelaius cyanopus* #334636, *Agelaius icterocephalus* #339772, *Agelaius phoeniceus* #341893, *Agelaius ruficapillus* #330775, *Agelaius thilius* #334615, *Agelaius xanthophthalmus* #324095, *Amblyranphus holoserius* #334662, *Cacicus cela* #324080, *Dolichonyx oryzivorus* #334721, *Euphagus cyanocephalus* #341985, *Gnorimopsar chopi* #334679, *Gymnomystax mexicanus* #339743, *Icterus galvula* #341938, *Icterus icterus* #324092, *Leister superciliaris* #334657, *Macroagelaius subalaris* #339783, *Molothrus bonariensis* #334768, *Nosopsar nigerrimus* #331150, *Oreopsar bolivianus* #334687, *Passerina cyaneus* #341743, *Psarocolius decumanus* #324065, *Psarocolius yuracares* #324076,

Pseudoleister guirahuro #330795, *Quiscalus mexicanus* #341975, *Quiscalus niger* #331153, *Quiscalus quiscula* #341733, *Scaphidura oryzivorus* #324097, *Spiza americana* MEX 2733, *Sturnella neglecta* #330039, *Thraupis episcopus* #339708, *Xanthopsar flavus* #330747, *Zonotrichia melodia* #341624; tissue sample without voucher specimen *Agelaius xanthomus* SML 86-1, *Agelaius humeralis*.

Louisiana State University Museum of Zoology (LSUMZ): *Agelaius tricolor* #130833, *Cacicus holosericeus* #98900, *Dives dives* #113959, *Lamprosars tanagra* #125586, *Sturnella bellicosa* LSUMZ B7737, *Xanthocephalus xanthocephalus* #126564.

American Museum of Natural History (AMNH): *Curaeus curaeus* APC 3295.