

## RECENT SPECIATION BETWEEN THE BALTIMORE ORIOLE AND THE BLACK-BACKED ORIOLE

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**Abstract.** A recent phylogenetic survey of the New World orioles (genus *Icterus*; Omland et al. 1999) suggested that the Baltimore Oriole (*I. galbula*) and the Black-backed Oriole (*I. abeillei*) are sister taxa. That survey examined mitochondrial DNA (mtDNA) from a single representative of each species in the genus. Here, we examine mtDNA sequences from 15 Black-backed and 20 Baltimore Orioles. The two species appear to be very recently diverged, with average sequence divergences for both cytochrome *b* (cyt *b*) and the control region indicating a probable late Pleistocene split. Despite this very recent divergence, there is one fixed base-pair difference between the species in cyt *b* and another in the control region, suggesting that one or both species have undergone a bottleneck during or since speciation. This molecular evidence of recent divergence suggests that male plumage differences between Black-backed and Baltimore Orioles evolved very rapidly.

**Key words:** *closely related species*, *Icterus galbula*, *Icterus abeillei*, *paraphyletic species*, *phylogeography*, *plumage evolution*, *speciation*.

### Especiación Reciente entre *Icterus galbula* y *Icterus abeillei*

**Resumen.** Un estudio filogenético reciente de los orioles del Nuevo Mundo (género *Icterus*; Omland, et al. 1999) sugiere que *Icterus galbula* e *I. abeillei* son taxa hermanos. En aquel estudio se examinó el ADN mitocondrial de un sólo representante de cada especie del género. En este estudio examinamos secuencias de ADN mitocondrial de 15 individuos de *I. abeillei* y 20 de *I. galbula*. Las dos especies parecen haber divergido recientemente con una separación promedio de las secuencias nucleotídicas en citocromo *b* y la región de control que indica que la divergencia ocurrió probablemente a fines del Pleistoceno. A pesar de haber divergido tan recientemente, existe una diferencia fija de un par de bases en la secuencia nucleotídica entre las dos especies en citocromo *b* y otra diferencia fija en la secuencia nucleotídica de la región de control, lo cual sugiere que una o ambas especies han sufrido un efecto de cuello de botella desde o durante el proceso

de especiación. Esta evidencia molecular de divergencia reciente sugiere que los elementos del plumaje en los machos evolucionaron muy rápidamente entre *I. abeillei* e *I. galbula*.

The Baltimore Oriole (*Icterus galbula*) and Bullock's Oriole (*I. bullockii*) hybridize in the American Midwest (Sibley and Short 1964), in one of the best-known hybrid zones in avian systematics. By contrast, only one putative hybridization has been documented between Bullock's and Black-backed Orioles (*I. abeillei*; Miller 1906). A recent expedition to Durango, Mexico, the site of Miller's report, found no orioles of either species (J. Rising, pers. comm.). The species status of all three taxa has changed repeatedly (AOU 1957, 1973, 1983, 1995, Sibley and Short 1964). The advent of molecular phylogenetic analyses has yielded surprising relationships among these species, especially given the marked differences in male plumage between Baltimore and Black-backed Orioles (Omland and Lanyon 2000).

Omland et al. (1999) included one individual from each of these three taxa in their phylogenetic survey of all members of *Icterus* using mtDNA. Surprisingly, they found the Baltimore Oriole individual to be closely related to the Black-backed Oriole (0.6% divergent in the combined data for the mitochondrial genes cyt *b* and ND2, Omland et al. 1999), whereas the Bullock's was most closely related to the members of the Streak-backed Oriole complex. Baltimore and Black-backed Orioles have never been known to hybridize, and have very disjunct breeding ranges (Howell and Webb 1995, Jaramillo and Burke 1999). Baltimore Orioles breed in eastern to midwestern North America and generally winter in Central and South America; thus they are long distance migrants (Kaufman 2000). Black-backed Orioles are resident in Mexico year-round (Fig. 1), but apparently are short-distance migrants within Mexico (Howell and Webb 1995).

Because the two Baltimore and Black-backed Orioles initially sequenced by Omland et al. (1999) were so closely related to each other, we decided that a closer examination of the genetic relationship between the species was warranted. If the mitochondrial relationship of the initial two Baltimore and Black-backed Orioles is supported when a larger number of samples from each species is examined, then one or both species must have undergone much more substantial plumage changes than have been observed in the other

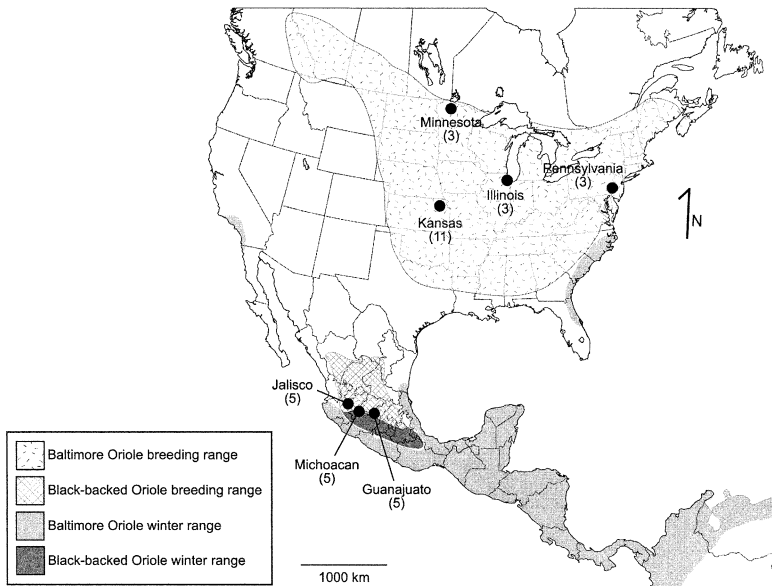


FIGURE 1. Breeding and wintering ranges of Baltimore Orioles and Black-backed Orioles (adapted from Jaramillo and Burke 1999). Baltimore Oriole breeding range is stippled; Black-backed Oriole breeding range is crosshatched; Baltimore winter range is light gray; and Black-backed winter range is dark gray. Dots indicate collection locations, and numbers indicate the number of samples taken at each location.

members of the genus *Icterus* (Omland and Lanyon 2000).

## METHODS

### SAMPLES

We sequenced 925 base pairs (bp) of cytochrome *b* and 344 bp of the control region from mitochondria of 20 Baltimore Orioles and 15 Black-backed Orioles (Fig. 1). We sequenced two close outgroups: one Bullock's Oriole and one Streak-backed Oriole (*I. pusillus*; Appendix). Additional *cyt b* outgroup sequences for Jamaican (*I. leucopteryx*), Altamira (*I. gularis*), Black-cowled (*I. prothemelas*), Orchard (*I. spurius spurius*), Spot-breasted (*I. pectoralis*), and Audubon's (*I. graduacauda*) Orioles had been obtained previously (Omland et al. 1999), and were selected to represent the three New World oriole clades.

### DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

We extracted DNA from muscle or feather tissue using the DNeasy Extraction Kit or the QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, California), and then amplified mitochondrial DNA from the *cyt b* gene and the control region. The amplification primers for *cyt b* were B1/L14990 (Kocher et al. 1989, referred to by the human mtDNA genome sequence number L14841) and HB4/H15916 (Lanyon 1994). We amplified the hypervariable Domain I of the control region using primers LGL2/L2263 and H417/H2607 (Tarr 1995). For sequencing we used the above primers, with the addition of two internal primers for *cyt b*: LCBA/L15350 (J. Klicka, pers. comm.) and HSH/H15424

(Hackett 1996). (The primer numbers refer to the location of the 3' ends of these primers on the chicken mitochondrial genome; Desjardins and Morais 1990.) Amplification typically involved an initial step of 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 45 sec, followed by a final extension of 72°C for 10 min (52°C for some control region amplifications), performed on either an MJ Research PTC-200 (MJ Research, Inc., Waltham, Massachusetts) or an Applied Biosystems GeneAmp® PCR System 9700 (Applied Biosystems, Inc., Foster City, California). We cleaned the products using the Qiagen QIAquick® PCR Purification Kit. Cycle sequencing reactions involved an initial step of 96°C for 5 min followed by 25 cycles of 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min on the equipment used for amplification, using Big Dye Chemistry (version 2, Applied Biosystems, Inc.). Sequencing products were cleaned using the recommended ethanol precipitation protocol, and sequenced on an Applied Biosystems/Hitachi ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Inc.). We aligned and edited the sequences using Sequencer™ 4.1 (Gene Codes 2000). Sequences have been deposited in GenBank (Appendix).

### PHYLOGENETIC ANALYSIS

We used PAUP\* (versions 4.0b8 and 4.0b10, Swofford 2002) for all analyses of *cyt b* and the control region. We computed uncorrected pairwise distances; additionally we computed Kimura's two-parameter corrected distances for *cyt b* (Kimura 1980). We performed maximum parsimony (MP), maximum likeli-

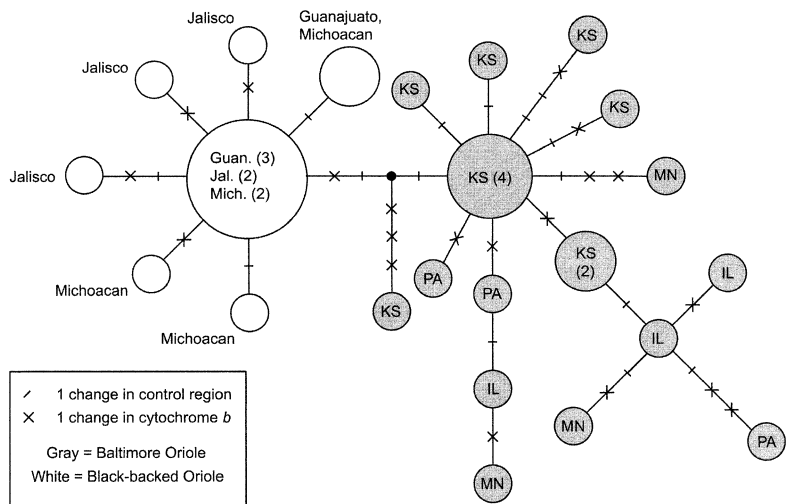


FIGURE 2. Unrooted haplotype network of combined control region and cytochrome *b* sequences for Baltimore Orioles (gray circles) and Black-backed Orioles (white circles). The diameter of each circle is proportional to the number of individuals with that haplotype. Collection locations are indicated within or next to each haplotype. Numbers in parentheses indicate number of individuals from a sampling location. One tick mark between haplotypes indicates that they differ by one base pair of the control region; one cross mark indicates a difference of one base pair in cytochrome *b*.

hood (ML), and distance (neighbor-joining [NJ]) analyses of both data sets, separately and combined. Where possible, we obtained starting trees for ML and distance analyses using stepwise addition. When that was not possible, we used NJ methods to obtain starting trees. For branch swapping, we used the TBR method (Swofford 2002). We used ModelTest (version 3.04, Posada and Crandall 1998) to determine which evolutionary model best fit our data, and then used that model to construct an ML tree in PAUP\*, using a heuristic search algorithm with 1000 random additions. We used the iterative method (Wilgenbusch and de Quieroz 2000) to find stable values for ti:tv ratio, shape parameter, and tree score for the ML tree. Alternate trees were created using the PAUP\* line editor, and examined to determine how many steps separated them from the shortest MP trees. We also used MacClade

(Maddison and Maddison 2000) to construct constraint trees for use in PAUP\* ML analyses. We used PAUP\* to conduct Shimodaira-Hasegawa comparisons (Shimodaira and Hasegawa 1999) of the best ML tree and the constrained ML trees.

RESULTS

Baltimore and Black-backed Orioles were separated by one fixed character difference in the 344 bp of the control region and one fixed difference in the 925 bp of *cyt b* studied (Fig. 2). There were fewer haplotypes per individual sampled in the Black-backed Orioles (seven haplotypes in fifteen individuals) than within Baltimore Orioles (16 haplotypes in 20 individuals).

We computed genetic distances for the control region and *cyt b*, calculated within as well as between species (Table 1). Uncorrected distances were the same

TABLE 1. Pairwise comparisons of the cytochrome *b* (925 bp, primers B1 to HB4) and control region (344 bp, primers LGL2 to H417) mtDNA sequences within and between Baltimore and Black-backed Orioles. Uncorrected and Kimura's two-parameter-corrected percent divergences were the same to two decimal places.

	Within Black-backed Oriole	Within Baltimore Oriole	Between species
Cytochrome <i>b</i>			
Average distance (%)	0.06	0.21	0.26
Minimum distance (%)	0.00	0.00	0.11
Maximum distance (%)	0.22	0.65	0.54
Control region			
Average distance (%)	0.16	0.40	0.74
Minimum distance (%)	0.00	0.00	0.29
Maximum distance (%)	0.58	1.16	1.45

as Kimura's two-parameter-corrected distances to the number of digits shown in the table. The average within-species distances between individuals in *cyt b* were 0.06% for Black-backed Orioles and 0.21% for Baltimore Orioles; whereas the average difference between the two species was 0.26%. Interestingly, the maximum within-species distances for *cyt b* were 0.22% and 0.65% (Black-backed and Baltimore Orioles, respectively), but between species the distance was 0.54%, less than the maximum distance within Baltimore Orioles.

ModelTest (Posada and Crandall 1998) results indicated that the HKY- $\Gamma$  model of evolution best fit our data. This model allows unequal base frequencies, two substitution types, and gamma-distributed rates (we used 10 discrete rate categories). We used this model for our maximum-likelihood analyses.

The phylogenetic trees we produced varied according to which mtDNA region was being analyzed and which analysis method was used, but generally showed that one of the two species was paraphyletic, with the other one nested within. For *cyt b*, ML analysis showed that Baltimore Oriole was nested within Black-backed Oriole (trees not shown), as did NJ. MP analysis produced multiple equally parsimonious trees, with either species being nested (trees not shown). The control region had the most variable results, but again one of the species was generally nested with respect to the other.

When the *cyt b* and control region data were combined, ML analysis showed Baltimore Oriole nested within Black-backed Oriole (Fig. 3). However, all 144 MP trees showed Black-backed Oriole nested within Baltimore Oriole (106 steps), as did NJ. Manual editing to create constraint trees in which both species were monophyletic, or Baltimore Oriole nested within Black-backed Oriole, produced trees that were only one step longer (107 steps). Although the best ML tree showed Baltimore Oriole nested within Black-backed Oriole, this topology did not differ significantly from trees in which Black-backed Oriole was constrained to be nested within Baltimore Oriole (Shimodaira-Hasegawa test,  $\text{diff } -\ln(l) = 1.59$ ,  $P = 0.36$ ) or trees in which both taxa were constrained to be reciprocally monophyletic (Shimodaira-Hasegawa test,  $\text{diff } -\ln(l) = 0.44$ ,  $P = 0.56$ ). Regardless of analysis method, all trees strongly supported the close mtDNA sister relationship of Baltimore and Black-backed Orioles: no individual of either taxon came out as distantly related.

## DISCUSSION

Baltimore and Black-backed Orioles differ by only one fixed difference in their *cyt b* sequences, and one fixed difference in their control region sequences. This is the smallest amount of difference that it is possible to have in each gene region and still diagnose the two taxa. We know of no other example of taxa so closely related yet with fixed nucleotide differences. Baltimore and Black-backed Orioles differ on average by only 0.26% in their *cyt b* sequences. A molecular clock of 1.4–1.6% divergence per million years (Fleischer et al. 1998) would place their divergence within roughly the last 150 000–200 000 years, a late Pleistocene divergence. The average control region divergence, 0.74%,

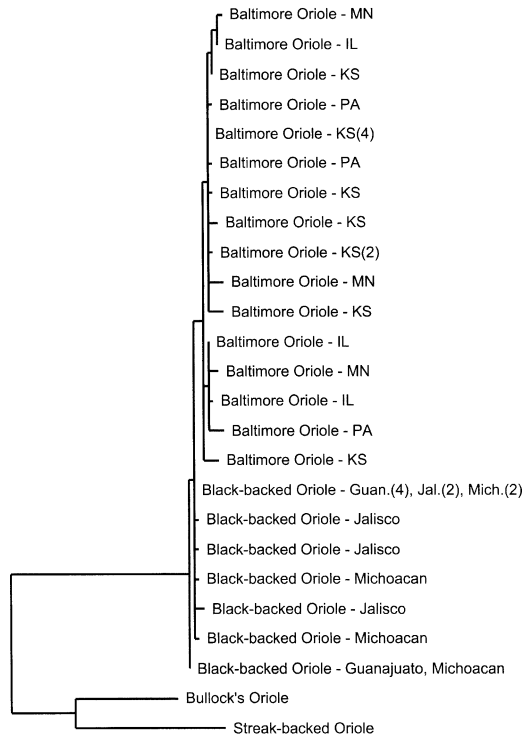


FIGURE 3. Phylogram constructed using combined cytochrome *b* and control-region data under maximum-likelihood criteria. The sampling locations for each haplotype are indicated by the name of the state, and when multiple individuals from a location share a haplotype, the number of individuals is indicated in parentheses. There was no bootstrap support for in-group nodes, although the node containing all Baltimore and Black-backed Orioles had 100% support.

provides another basis for examining very closely related species and suggests an even more recent divergence (Quinn 1992 calibration: 35 000 years; Lambert et al. 2002 calibration: 5000 years). Although we view the divergence-time estimates as imprecise, the amount of molecular divergence is less than the divergence for every species pair reported by Klicka and Zink (1997) except for the Brewer's and Timberline Sparrows (*Spizella breweri*), and less than the divergence for all 41 pairs of subspecies reported by Avise and Walker (1998). Other recently diverged bird species include the ground finches of the Galapagos (Sato et al. 1999), *Parula* warblers (Lovette and Bermingham 2001), Mallards (*Anas platyrhynchos*) and Australian Black Ducks (*Anas rubripes*; Avise et al. 1990). Regardless of when these two oriole species diverged, they are more recently diverged than almost all diagnosable avian taxa.

Often, when taxa are recently diverged, their mtDNA haplotypes are still intermixed with each other (polyphyly, e.g., Baker et al. 2003, Funk and Omland 2003). Our analyses are inconclusive regarding the



monophyly, paraphyly, or polyphyly of Baltimore and Black-backed Orioles. Rooting trees containing such recently diverged species will be a general problem, so that mitochondrial monophyly of very closely related species may be difficult to verify or reject (cf. Zink 2004). Therefore, in such cases it will be difficult to apply the criterion of mitochondrial monophyly to assess species limits. The fixed mtDNA differences between Baltimore Orioles and Black-backed Orioles are surprising in species so recently diverged. These fixed differences suggest that one or both taxa may have undergone a bottleneck during or since speciation. An alternative explanation for the low amount of molecular divergence between the two species is hybridization followed by a bottleneck or selective sweep. We are currently examining the relationship between these taxa using data from multiple nuclear introns, although nuclear introns accumulate fixed differences more slowly than mtDNA (Moore 1995). Preliminary intron sequence data (Kondo et al., unpubl. data) support the hypothesis of very recent divergence.

Omland and Lanyon (2000) examined male plumage evolution across all New World orioles. They found 44 characters that differed across the genus. They compared plumage character differences to mtDNA divergence for each species pair. Generally, maximal plumage difference correlated with maximal mtDNA divergence. Notably, the Baltimore–Black-backed species pair did not follow this trend. Sampling one Baltimore Oriole and one Black-backed Oriole had suggested that they are each other's closest relatives by mtDNA (Omland et al. 1999), yet they differed in 17 plumage characters. Our results from 33 more individuals confirm the close mtDNA relatedness of these species. The amount of plumage divergence is much greater than one might anticipate, given their close mitochondrial relationship. The rapid divergence in male plumage between Baltimore and Black-backed Orioles could have several causes, including sexual selection or possibly reproductive character displacement. We know of few other examples of bird taxa that are so recently diverged with so many plumage differences (Avise et al. 1990). Additionally, rapid changes in migratory distance have only rarely been documented (Able and Belthoff 1998). Our finding of very recent divergence between Baltimore and Black-backed Orioles makes them an ideal system to further examine the relationships between speciation, migration, and plumage evolution.

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APPENDIX. Museum catalog numbers, GenBank accession numbers, and collection localities for four species of orioles analyzed in this study.

Catalog number <sup>a</sup>	GenBank accession numbers		Collection locality
	Control region	Cyt <i>b</i>	
Black-backed Oriole			
MZFC keo27	AY607589	AY607604	Mexico, Guanajuato, Salvatierra
MZFC keo28	AY607590	AY607605	Mexico, Guanajuato, Salvatierra
MZFC keo29	AY607591	AY607606	Mexico, Guanajuato, Salvatierra
MZFC keo30	AY607592	AY607607	Mexico, Guanajuato, Yuriria
MZFC keo31	AY607593	AY607608	Mexico, Guanajuato, Yuriria
MZFC keo32	AY607594	AY607609	Mexico, Jalisco, Chapala
MZFC keo33	AY607595	AY607610	Mexico, Jalisco, Chapala
MZFC keo34	AY607596	AY607611	Mexico, Jalisco, Chapala
MZFC keo35	AY607597	AY607612	Mexico, Jalisco, Chapala
MZFC keo37	AY607598	AY607613	Mexico, Jalisco, Chapala
MZFC keo45	AY607599	AY607614	Mexico, Michoacán, Briseñas de Matamoros
MZFC keo46	AY607600	AY607615	Mexico, Michoacán, Briseñas de Matamoros
MZFC keo47	AY607601	AY607616	Mexico, Michoacán, Briseñas de Matamoros
MZFC keo48	AY607602	AY607617	Mexico, Michoacán, Briseñas de Matamoros
MZFC keo49	AY607603	AY607618	Mexico, Michoacán, Briseñas de Matamoros
Baltimore Oriole			
FMNH 395866	AY607637	AY607653	USA, Illinois, Cook Co.
FMNH 350604	AY607621	AY607656	USA, Illinois, Cook Co.
FMNH 394643	AY607636	AY607658	USA, Illinois, Cook Co.
UKNHM 90711	AY607630	AY607643	USA, Kansas, Douglas Co.
UKNHM 90707	AY607627	AY607652	USA, Kansas, Jefferson Co.
UKNHM 90706	AY607632	AY607645	USA, Kansas, Jefferson Co.
UKNHM 90708	AY607628	AY607641	USA, Kansas, Jefferson Co.
UKNHM 90704	AY607631	AY607644	USA, Kansas, Jefferson Co.
UKNHM 90702	AY607629	AY607642	USA, Kansas, Jefferson Co.
UKNHM 90710	AY607633	AY607646	USA, Kansas, Jefferson Co.
UKNHM 90705	AY607634	AY607647	USA, Kansas, Jefferson Co.
UKNHM 90701	AY607635	AY607648	USA, Kansas, Jefferson Co.
UKNHM 90703	AY607620	AY607649	USA, Kansas, Jefferson Co.
UKNHM 90714	AY607638	AY607654	USA, Kansas, Jefferson Co.
BMNH X7763	AY607626	AY607640	USA, Minnesota, Beltrami Co.
BMNH X7799	AY607619	AY607657	USA, Minnesota, Chisago Co.
BMNH X7630	AY607625	AY607651	USA, Minnesota, Hennepin Co.
ANSP 10126	AY607622	AY607655	USA, Pennsylvania, Bucks Co.
ANSP 10134	AY607623	AY607639	USA, Pennsylvania, Bucks Co.
ANSP 10148	AY607624	AY607650	USA, Pennsylvania, Bucks Co.
Bullock's Oriole			
UWBM 55975	AY611475	AY611476	USA, Washington, Douglas Co.
Streak-backed Oriole			
MZFC keo38	AY611477	AY611478	Mexico, Jalisco, Chapala

<sup>a</sup> ANSP = Academy of Natural Sciences, Philadelphia; BMNH = J.F. Bell Museum of Natural History, University of Minnesota; FMNH = Field Museum of Natural History; MZFC = Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autónoma de México; UWBM = University of Washington, Burke Museum; and UKNHM = University of Kansas Natural History Museum.