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USING HISTORICAL DNA TO CHARACTERIZE HYBRIDIZATION BETWEEN BALTIMORE ORIOLES (*ICTERUS GALBULA*) AND BULLOCK'S ORIOLES (*I. BULLOCKII*)

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ABSTRACT.—Studies of genetic variation across hybrid zones have demonstrated that the evolutionary dynamics within them are often complicated. Using DNA extracted from toe pads of 701 individuals collected by Sibley and Short (1964) about 50 years ago from across the *Icterus bullockii–I. galbula* hybrid zone, we calculated mitochondrial cline shape parameters and compared them with plumage-based inferences of the hybrid-zone structure. Genetic and hybrid index score clines, estimated from populations collected along the Platte River in Nebraska, were both concordant (equal widths) and coincident (same center). More generally, the proportion of *I. bullockii* haplotypes within a sampling locality was strongly and significantly correlated with mean hybrid index scores across Colorado, Kansas, Nebraska, Oklahoma, and South Dakota. The relatively narrow width of the mitochondrial cline (328 km) indicates selection against hybridization, which may be mediated through differences in either molt and migration schedules or thermoregulatory capabilities (or both) of the parental species. Our results provide the first robust historical genetic characterization of this avian hybrid zone, laying the foundation for more in-depth investigations of temporal patterns of gene flow and introgression. *Received 6 July 2010, accepted 2 October 2010.*

Key words: clinal variation, hDNA, hybrid zones, speciation.

Uso de ADN Histórico para Caracterizar la Hibridación entre *Icterus galbula* e *I. bullockii*

RESUMEN.— Los estudios de la variación genética a través de las zonas de hibridación han demostrado que las dinámicas evolutivas que suceden en éstas a menudo son complicadas. Empleando ADN extraido de los dedos de 701 individuos coleccionados en la zona de hibridación entre *Icterus galbula* e *I. bullockii* por Sibley y Short (1964) hace cerca de 50 años, calculamos los parámetros de forma de la clina mitocondrial y los comparamos con inferencias sobre la estructura de la zona de hibridación basadas en el plumaje. Las clinas genéticas y del puntaje de un índice de hibridación basado en el plumaje, estimadas a partir de colecciones hechas en poblaciones a lo largo del río Platte en Nebraska, fueron tanto concordantes (igual ancho) como coincidentes (igual centro). De modo más general, la proporción de haplotipos de *I. bullockii* en una localidad de muestreo se correlacionó fuerte y significativamente con los puntajes promedio del índice a través de Colorado, Kansas, Nebraska, Oklahoma y South Dakota. La relativa estrechez de la clina mitocondrial (ancho de 328 km) indica selección en contra de la hibridación, que podría ser mediada por las diferencias en los cronogramas de muda y migración y/o en las habilidades de termorregulación de las especies parentales. Nuestros resultados brindan la primera caracterización genética robusta de carácter histórico sobre esta zona de hibridación de aves y representan una base para realizar investigaciones más profundas acerca de los patrones temporales de flujo genético e introgresión.

HYBRID ZONES OFFER tractable opportunities for investigating the evolutionary processes important in creating and maintaining biological diversity (Hewitt 1988, Harrison 1990, Arnold 1997, Rieseberg and Carney 1998, Jiggins and Mallet 2000, Mallet 2005). Since Haldane (1948) first outlined the mathematical framework that became the basis for modern cline analyses (Endler 1977; Szymura and Barton 1986, 1991), researchers have developed increasingly powerful analytical tools that glean information about the speciation process from hybrid-zone structure. In particular, cline-based analyses have been used to investigate the strength of selection operating against hybrids in naturally occurring hybrid zones (Mallet and Barton 1989, Mallet et al. 1990, Porter et al. 1997).

There is a cluster of naturally occurring contact zones in the Great Plains of North America where a variety of closely related avian species pairs form a "suture zone" (Remington 1968, Rising 1983a), which provides a natural laboratory (Hewitt 1988) for investigating the evolutionary and ecological processes that are important in maintaining isolation between avian species. Although they are not sister species (Omland et al. 1999, Jacobsen et al. 2010), *Icterus galbula* (Baltimore Oriole) and *I. bullockii* (Bullock's Oriole) form a hybrid zone within the Great Plains suture zone that is particularly amenable to investigations of the structure of avian hybrid zones.

Both species breed in riparian woodlands, but they have parapatric breeding ranges (Fig. 1): Baltimore Orioles are found primarily in eastern North America, whereas Bullock's Orioles breed in western North America (Sibley and Short 1964, Rising 1983b). Their breeding ranges overlap in a region that is ~240 km wide and extends 2,700 km north to south from Saskatchewan to central Texas (Sibley and Short 1964, Rising 1983b). The first in-depth study of the zone was conducted by Sibley and Short (1964), who collected a large series of specimens from within and near the zone and used that material to examine geographic variation in plumage and mensural characters. Although Sibley and Short (1964) collected specimens from 39 locations throughout the hybrid zone (Fig. 1), most of their efforts focused on the Platte and Niobrara River valleys, which run west to east across the hybrid zone in southern Nebraska and Colorado, and northern Nebraska, respectively. They scored 701 birds (see online Appendix) for a variety of plumage and mensural characteristics. On the basis of the resulting gradient of observed hybrid index scores across the hybrid zone, they suggested (1) that these two forms had only recently come into secondary contact and (2) that hybrid individuals face no fitness disadvantage. On the basis of their interpretations, they argued that *I. galbula* and *I. bullockii* should be merged taxonomically into a single biological species (Sibley and Short 1964; see Brelsford and Irwin 2009 for a discussion of a similar situation in Yellow-rumped Warblers [*Dendroica coronata*]).

By contrast, Rising (1969, 1996) argued that these two orioles should be considered separate species partly on the basis of differences in thermoregulation. Rising (1969) observed that if *I. galbula* and *I. bullockii* were experimentally exposed to equally hot environments in the laboratory, *I. galbula* became more stressed, resulting in increased metabolic rate and evaporative water loss. Moving west from the center of the hybrid zone, the environment becomes more xeric, potentially limiting the spread of alleles from *I. galbula* to *I. bullockii*. Similar selection pressures may occur in the east, preventing *I. bullockii* alleles from spreading into the more mesic environment inhabited by *I. galbula*. Beyond these differences in thermoregulatory capacity, Rising (1970) also suggested that the phenotypic variation across the hybrid zone approximated a step-cline consistent with a scenario of comparatively high hybrid fitness only within the intermediate environment found in the center of the zone and strong selection against hybrids on either side.

Rohwer and Manning (1990) suggested that differences between *I. galbula* and *I. bullockii* in molt and migration schedules could also contribute to selection against hybrids. At the end of the breeding season, *I. bullockii* migrate to the southwestern United States and Mexico, where they undergo prebasic molt. By
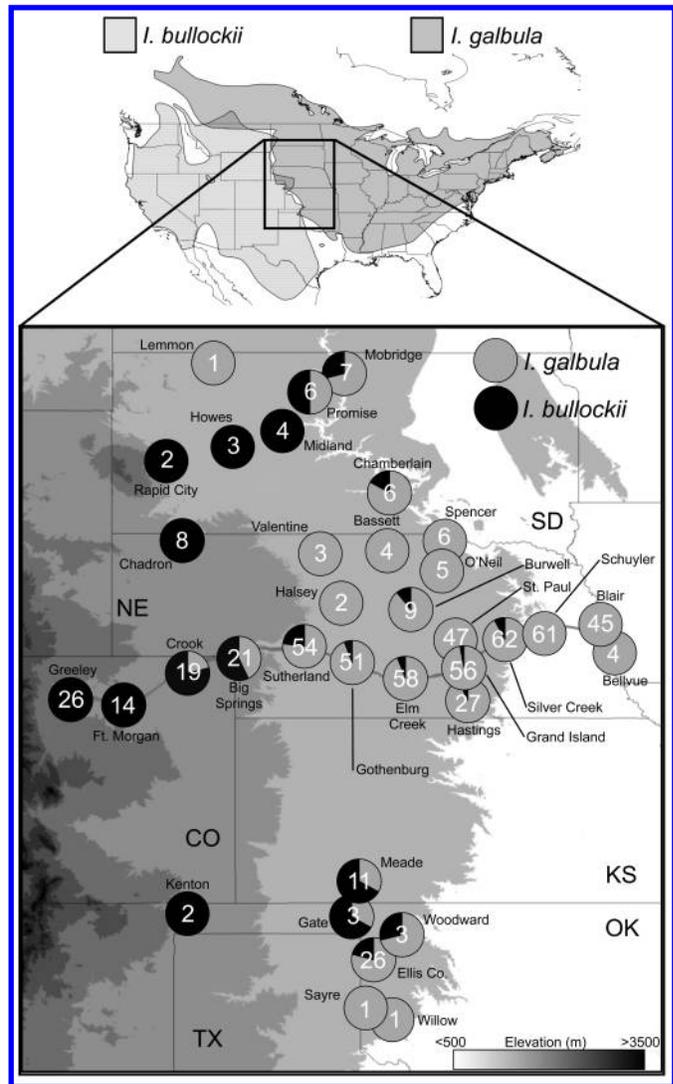

Fig. 1. Breeding distributions, sampling localities, and mitochondrial haplotype compositions for populations of *Icterus bullockii* and *I. galbula*. Numbers of individuals sampled and haplotype frequencies are for males only. The Platte River transect extends from Greeley, Colorado, in the west to Blair, Nebraska, in the east. Digital maps (Ridgely et al. 2003) were downloaded from NatureServe (2006) and modified.

contrast, *I. galbula* undergo prebasic molt before they migrate to their winter range in Mexico and Central America. Although the schedule of molt and migration in hybrids is unknown, Rohwer and Manning (1990) suggested that the difference in scheduling of molt and migration between the parental species could result in reduced survival or reproductive success in hybrids because hybrid individuals may attempt a prebasic molt both before and after migration or a very late molt after delayed migration. Rohwer and Manning (1990) contended that both environmental variation (*sensu* Rising 1969, 1970) and potential problems in balancing molt and migration serve to reduce the fitness of hybrid individuals.

Here we provide a historical genetic framework for the well-studied hybrid zone between Baltimore and Bullock's orioles by

characterizing the genetic structure of the zone as it existed ~50 years ago, using DNA extracted from the specimens collected by Sibley and Short (1964). Our investigation had three primary goals: (1) to develop a robust method to quickly identify oriole mitochondrial haplotypes from museum specimens; (2) to estimate the center and width of the mitochondrial and plumage clines along the Platte River transect; and (3) to compare the genetic and phenotypic composition of populations sampled from across the hybrid zone.

Methods

Sampling.—We obtained tissues from modern reference specimens from allopatric populations of *I. bullockii* (Washington) and *I. galbula* (New York) for use in designing the species-specific restriction enzyme assay (see below). We collected historical samples from toe pads, using single-use razor blades, of the 701 specimens collected by Sibley and Short (1964) that are housed in the Ornithology Collection of the Cornell University Museum of Vertebrates. Detailed locality information for all individuals is available in the online Appendix.

RFLP assay.—Using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California), we extracted DNA from seven *I. bullockii* tissues and seven *I. galbula* tissues following the manufacturer's recommended protocol, for use in designing a restriction digest assay capable of reliably assigning a mitochondrial haplotype as belonging to either *I. bullockii* or *I. galbula*. We amplified the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) by polymerase chain reaction (PCR) using primers METb and TRPc (Hunt et al. 2001) in 10 μL containing 5.5 μL sterile H_2O, 4 mM $MgCl_2$, 1–10 ng DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.25 μM of each primer (METb and TRPc), 0.25 mM dNTPs (0.06 mM each of dATP, dCTP, dGTP, and dTTP), and 25 U Jumpstart *Taq* DNA polymerase (Sigma). We used the following PCR amplification profile: 95°C for 4 min 30 s, then 30 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min 20 s, and a final, one-time cycle of 72°C for 4 min 30 s.

The PCR amplicons were cleaned using Exo-Sap and cycle-sequenced in both directions (using the amplification primers) using the Big Dye Terminator Cycle-Sequencing Kit, version 3.1 (Applied Biosystems, Foster City, California). We edited and assembled sequences using SEQUENCHER, version 4.7 (GeneCodes, Ann Arbor, Michigan), and GENEIOUS, version 4.8 (Drummond et al. 2009).

Using the ND2 sequences (GenBank accession nos. HQ384241–HQ384254) from the seven representatives of *I. bullockii* and *I. galbula*, respectively, we identified a diagnostic EcoRV restriction cut site (5′ GAT″ATC 3′) difference between the species (*I. bullockii*: 5′ GATATC 3′, *I. galbula*: 5′ GATTTC 3′), such that following a restriction digest of ND2 with EcoRV, *I. bullockii* individuals show two bands and *I. galbula* individuals a single band. We then designed primers (IcterusND2EcoRV. For: 5′ TCAAGCAACTGCCTCTGCCCTTGT 3′ and Icterus ND2EcoRV.Rev: 5′ AGCCTTGGAGGACTTCTGGGAAT 3′) to amplify a smaller portion (179 base pairs [bp]) of the ND2 gene that encompassed this diagnostic restriction site (bp 5495 in the *Gallus gallus* reference sequence, GenBank NC_001323). After optimizing amplification conditions for these primers (see below)

and verifying the utility of the restriction site assay to identify the mitochondrial haplotype of each of our 14 modern specimens, we added a fluorescent label to each primer so that we could assay the presence or absence of the three potential postdigestion fragments using an Applied Biosystems 3100 Genome Analyzer and the GeneMapper software package.

Extraction and amplification of DNA from museum specimens.—We extracted DNA from each toe pad in a dedicated historical DNA (hDNA) lab, taking care to avoid contamination. All toe-pad extractions were done in a laminar-flow workstation (Purifier Horizontal Clean Bench, Labconco, Kansas City, Missouri) cleaned both before and after use with a 10% bleach solution. Additionally, all surfaces in the workstation were exposed to UV light for at least 30 min after each workday. We extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen), with two minor modifications to the manufacturer's protocol. First, we added 40 μL, instead of 20 μL, proteinase K during the initial digestion step. Second, we performed two elutions using 50 μL each time, for a total elution volume of 100 μL. We extracted DNA from toe pads in batches of 24 samples and included a negative extraction control in each batch.

Like the extractions, all PCR amplifications of hDNA samples were set up under a laminar flow hood in a dedicated hDNA lab. We performed hDNA amplifications in batches of 24 samples and always included a negative extraction control and negative PCR control. Each hDNA sample was amplified in a 10-μL reaction containing 1–10 ng of hDNA, 5.5 μL sterile H_2O, 2.5 mM dNTPs (0.6 mM each of dATP, dCTP, dGTP, and dTTP), 0.15 μM of each flourescently labeled primer (IcterusND2EcoRV.For and Icterus ND2EcoRV.Rev), 10mM Trizma HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, and 25 U Jumpstart *Taq* DNA polymerase (Sigma). We amplified the 179 bp portion of ND2 using the following profile: 95°C for 4 min 30 s, then 50 cycles of 95°C for 45 s, 50°C for 45 s, 72°C for 1 min, and a final, one-time cycle of 72°C for 4 min 30 s.

To determine whether amplification was successful, we electrophoresed 2 μL of each PCR amplicon on a 1% TAE agarose gel. Successful amplifications were then restriction digested using EcoRV in a 20-μL reaction containing DNA grade water, 10 mM NaCl, 5 mM Tris-HCl pH 7.9, 1 mM $MgCl_2$, 0.1 mM DTT, and 0.001 U μL^{-1} EcoRV restriction enzyme (New England BioLabs, Ipswich, Massachusetts) added to the remaining 8 μL of PCR product. Digests were performed for 12 h at 37°C.

Genotyping.—DNA fragment sizes of digested samples were determined using an ABI 3100 Genetic Analyzer (Applied Biosystems) with a GeneScan^T-500 Liz size standard (Applied Biosystems), then visualized using GENEMAPPER, version 3.7 (Applied Biosystems). Samples were then scored by visual examination as either *I. bullockii* (two bands, 67 and 112 bp in length) or *I. galbula* (one band, 179 bp in length).

Analyses.—For two reasons, we estimated cline shape parameters using only the samples collected along the Platte River transect: (1) this transect is naturally roughly linear and therefore particularly amenable to clinal analysis; and (2) Sibley and Short (1964) focused most of their collecting efforts on the Platte River transect and, hence, sample sizes from this region are particularly robust.

In the clinal analysis, the westernmost sampling locality along the Platte River transect (Greeley, Colorado; Fig. 1) was set to 0 km. The distance between Greeley and each subsequent location

Table 1. Sampling localities, sample sizes, mitochondrial DNA haplotype composition, and mean hybrid index scores for populations sampled by Sibley and Short (1964).

Locality	Latitude	Longitude	n (males)	Proportion of *I. bullockii* haplotypes (males only)	Mean hybrid index score (males only)[a]	Distance along Platte River transect (km)
Greeley, Colorado	40.35	−104.82	26	1.00	11.23	0
Fort Morgan, Colorado	40.27	−103.94	14	1.00	10.92	55
Crook, Colorado	40.85	−102.71	19	0.79	*9.44*	176
Big Springs, Nebraska	40.98	−102.18	21	0.43	*7.39*	222
Sutherland, Nebraska	41.14	−101.11	54	0.23	*3.65*	314
Gothenburg, Nebraska	40.90	−100.15	52 (51)	0.04	*2.88*	399
Elm Creek, Nebraska	40.69	−99.34	59 (58)	0.05	*2.53*	472
Grand Island, Nebraska	40.84	−98.44	67 (56)	0.03	*2.23*	549
Silver Creek, Nebraska	41.28	−97.69	63 (62)	0.02	*2.29*	629
Schuyler, Nebraska	41.42	−97.04	62 (61)	0.00	1.55	686
Blair, Nebraska	41.52	−96.07	45	0.00	1.18	767
Chadron, Nebraska	42.85	−102.96	9 (8)	1.00	10.88	—
Meade, Kansas	37.28	−100.12	16 (11)	0.73	*5.77*	—
Ellis County, Oklahoma	36.13	−99.78	31 (27)	0.06	*4.56*	—
Chamberlain, South Dakota	43.74	−99.50	6	0.17	*2.83*	—
Spencer, Nebraska	42.81	−98.65	7 (6)	0.00	*2.00*	—
St. Paul, Nebraska	41.17	−98.46	51 (47)	0.00	*2.50*	—
Hastings, Nebraska	40.46	−98.39	31 (27)	0.07	*2.48*	—
Rapid City, South Dakota	44.15	−103.23	3 (2)	1.00	—	—
Kenton, Oklahoma	36.88	−102.90	2	1.00	—	—
Lemmon, South Dakota	45.80	−102.40	1	0.00	—	—
Howes, South Dakota	44.51	−101.96	4 (3)	1.00	—	—
Midland, South Dakota	44.69	−101.18	4	1.00	—	—
Promise, South Dakota	45.27	−100.90	7 (6)	0.50	—	—
Valentine, Nebraska	42.57	−100.66	4 (3)	0.00	—	—
Mobridge, South Dakota	45.65	−100.34	7	0.29	—	—
Halsey, Nebraska	41.95	−100.28	2	0.00	—	—
Gate, Oklahoma	36.91	−100.05	5 (3)	0.67	—	—
Sayre, Oklahoma	35.37	−99.64	1	0.00	—	—
Bassett, Nebraska	42.72	−99.59	4	0.00	—	—
Willow, Oklahoma	35.08	−99.51	1	0.00	—	—
Woodward, Oklahoma	36.46	−99.45	4 (3)	0.33	—	—
Burwell, Nebraska	41.76	−99.26	10 (9)	0.11	—	—
O'Neill, Nebraska	42.45	−98.68	5	0.00	—	—
Bellvue, Nebraska	41.15	−95.88	4	0.00	—	—

[a] Mean hybrid index scores are included only for populations with at least 5 males measured by Sibley and Short (1964). Where the value is in italics, the populations were included in both Pearson correlation analyses.

to the east was then measured using Google Earth. This resulted in a 767-km linear transect (the Platte River transect) from Greeley to Blair, Nebraska (Table 1). Within each location along this transect, each individual was given a binary score based on its mitochondrial haplotype: 0 for *I. galbula* or 1 for *I. bullockii.*

We used the program CLINEFIT to analyze the mitochondrial data (Porter et al. 1997). CLINEFIT investigates patterns of introgression along a sampling transect using methods developed by Szymura and Barton (1986, 1991) to estimate cline shape parameters (here, center and width) that explore the relationship between the geographic location of the sampling localities and allele frequency data within each locality. We also estimated shape parameters for the plumage cline, using the mean hybrid index score (for males only; see below) as determined by Sibley and Short (1964) for each sampling locality. Prior to analysis, we scaled their hybrid index scores from 0 to 1, with 0 representing the lowest mean population score (1.18; Blair) and 1 representing

the highest (11.23; Greeley). We estimated (1) the center and width of the mitochondrial DNA cline along the Platte River transect and (2) two likelihood support limits ($\ln L_{max} - 2$, to assess support; these are analogous to 95% confidence intervals; Edwards 1972) using CLINEFIT with the following search parameters: burn-in: parameter tries per step – 300; sampling for support; replicates saved – 4,000; and 30 replicates per saves. Using the same search parameters, we estimated the center and width of the plumage cline, but we do not report log likelihood values because the CLINEFIT likelihood equations assume a genetic model with binomial variance, which is violated by our plumage data. We determined whether the mitochondrial DNA cline and the plumage cline had equal centers (coincidence) and equal widths (concordance) by assessing whether or not the center and width estimates for the plumage cline fell within the log-likelihood support limits for the center and width estimates for the mitochondrial cline.

Using data from males only, we performed two Pearson correlation analyses to compare the genetic (proportion of *I. bullockii* mitochondrial haplotypes in a population) and phenotypic composition (mean hybrid index score) of the sampled populations. The first included all populations for which there were at least five individuals sampled by Sibley and Short (1964). Because the correlation between genotype and phenotype should be strong on either side of the hybrid zone, where presumably only pure parentals are present, we performed a second correlation analysis that included only populations between Crook, Colorado, and Silver Creek, Nebraska, inclusively (Table 1). These analyses were performed at the population (rather than individual) level because Sibley and Short (1964) reported hybrid index scores for populations only. Many fewer females (42) than males (659) were collected, and, because the hybrid index scoring system differs between the sexes, females were excluded from these analyses.

For each male, Sibley and Short (1964) assessed whether the specimen had *I. galbula*, *I. bullockii*, or intermediate plumage patterns for three body regions (head, wing, and tail). Within each region, they scored multiple characters (e.g., head pattern consisted of scores for superciliary, forehead, sides of neck, ear coverts, and throat characters) but standardized the scores for each region on a scale of 0–4. The hybrid index score for an individual was the sum of the subtotals for each body region, such that the scores ranged from zero (putatively pure *I. galbula*) to 12 (putatively pure *I. bullockii*). For those localities with at least five sampled males, population mean hybrid index scores are given in Table 1.

RESULTS

We were able to assay the mitochondrial haplotype of 686 of the 701 (98%) *Icterus* individuals collected and analyzed by Sibley and Short (1964). Of the 686 individuals that we scored, 131 had *I. bullockii* mitochondrial haplotypes and the remaining 555 had *I. galbula* mitochondrial haplotypes (online Appendix). The proportion of *I. bullockii* haplotypes in populations sampled along the Platte River ranged from 1.0 (Greeley and Ft. Morgan, Colorado) to 0.0 (Schuyler and Blair, Nebraska; Fig. 1).

Using the mitochondrial haplotype data and the mean male hybrid index score for populations sampled along the Platte River transect (Table 1 and Fig. 1), we compared the genetic and plumage clines (Fig. 2). The mitochondrial cline was estimated to be 328 km wide (two likelihood support limits: 268–406 km), centered 240 km east of Greeley (two likelihood support limits: 206–270 km). The plumage cline was both concordant and coincident with the mitochondrial cline, in that the width (276 km) and center (236 km east of Greeley) estimates were within the two likelihood support limits of the mitochondrial cline parameter estimates. Both clines are centered between the Big Springs and Sutherland, Nebraska, sampling localities (Fig. 1).

Extending the comparison between the genetic and phenotypic composition of sampled populations to all localities for which Sibley and Short measured at least 5 males and reported a mean hybrid index score (Table 1) resulted in a significant correlation between mitochondrial haplotypes and male plumage patterns ($r = 0.969$, $t = 15.82$, df = 16, $P < 0.001$; Fig. 3). The correlation was also significant when we excluded the peripheral localities ($r = 0.912$, $t = 7.36$, df = 11, $P < 0.001$; Fig. 3).
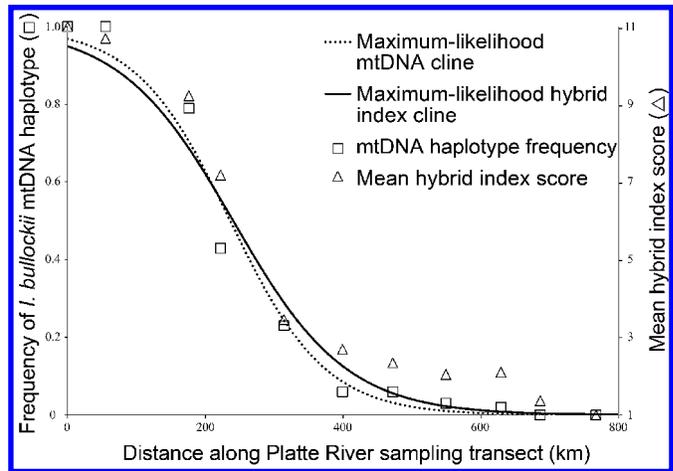

FIG. 2. Maximum-likelihood clines in mitochondrial haplotype frequencies and mean hybrid index score plotted against geographic location of sampled populations along the Platte River transect. Both clines were estimated using data from males only (see text).

DISCUSSION

Using DNA extracted from toe pads sampled from the specimens collected by Sibley and Short (1964) in their characterization of the *I. bullockii–I. galbula* hybrid zone, we were able to successfully generate mitochondrial haplotype data from nearly all individuals. In doing so, we produced the first robust genetic characterization of the *Icterus* hybrid zone as it stood ~50 years ago, thereby establishing a baseline for future work investigating the temporal dynamics of gene flow and introgression across this avian hybrid zone.
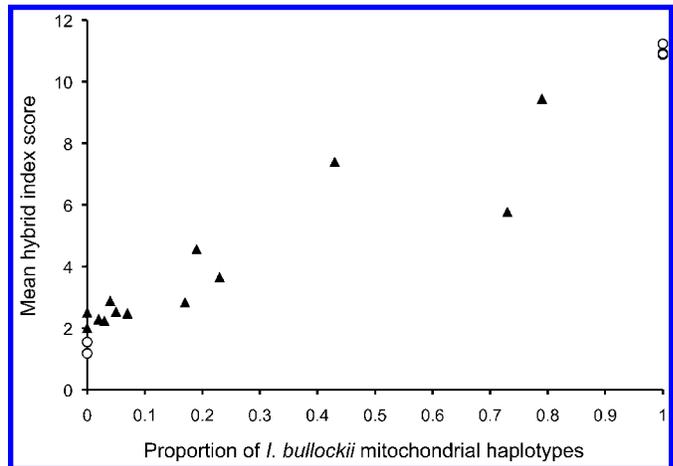

FIG. 3. Correlation between mitochondrial haplotype frequencies and mean hybrid index score for populations from which data were available from at least 5 males. Closed triangles represent the sample when peripheral (open circles) populations were excluded. Both correlations were significant (all populations: $t = 15.82$, df = 16, $P = 3.44e\text{-}11$, $r = 0.969$; central populations only: $t = 7.36$, df = 11, $P = 1.43e\text{-}05$, $r = 0.912$).

We found strong agreement between our genetic characterization of the *I. bullockii*–*I. galbula* hybrid zone and the characterization made by Sibley and Short (1964), which were primarily based on plumage patterns of the same individuals (Figs. 2 and 3). Estimates of cline shape parameters were both coincident and concordant using our significance criteria of whether the point estimates for the plumage cline fell within the two-likelihood support limits of the mitochondrial cline. A qualitatively similar pattern of plumage and mitochondrial concordance was reported recently for the hybrid zone between *Pheucticus melanocephalus* (Black-headed Grosbeak) and *P. ludovicianus* (Rose-breasted Grosbeaks), two species that also hybridize in the Great Plains of North America (Mettler and Spellman 2009), although that study did not test formally for coincidence and concordance between the clines they estimated from plumage-based hybrid index scores and from mitochondrial haplotypes. In addition, a recent characterization of the hybrid zone between MacGillivray's Warblers (*Oporornis tolmiei*) and Mourning Warblers (*O. philadelphia*) found strong concordance between morphological and molecular assessments of hybridization (Irwin et al. 2009). Nonetheless, this pattern of plumage–mitochondrial DNA concordance in the orioles is not a trivial result, as other studies of avian hybrid zones have found differential introgression of genetic and plumage characters that have informed hypotheses about the selective forces that have shaped those hybrid zones (Brumfield et al. 2001, Rohwer et al. 2001).

Sibley and Short (1964) defined the oriole hybrid zone as encompassing populations with mean hybrid index scores from 3.0 to 9.0, and they estimated it to be approximately 240–320 km wide. Their estimate is notably similar to the widths we derived using more recently developed cline analysis techniques for both the plumage (276 km) and mitochondrial (328 km) clines. Comparably, they predicted the center of the hybrid zone to be 24 km east of Big Springs, Nebraska, which places their center ~246 km from Greeley, almost precisely where we also estimated that both clines were centered (mitochondrial: 240 km east of Greeley; plumage: 236 km east of Greeley).

Given that *I. bullockii* and *I. galbula* are not sister species (Omland et al. 1999, Jacobsen et al. 2010), the hybrid zone between them had to have formed through secondary contact (Rising 1983b, 1996) of previously allopatric populations. In the absence of selection, the width (*w*) of such a cline is determined by the length of time since contact (*t*) and the root mean square (RMS) dispersal distance (σ): $w = 2.51\sigma\sqrt{t}$ (Barton and Gale 1993). It is important to note that this model assumes random mating within the hybrid zone, a condition that exists, evidently, because mating within the zone is not assortative (Rising 1983a, b). Further, we assume one generation per year (Rising and Flood 1998, Rising and Williams 1999), and so time since contact and RMS dispersal distances are measured in years and per year, respectively. We do not have accurate estimates of either the time since contact between these species or the RMS dispersal distance. However, if we assume that the current hybridization between these species began following the warming trend that allowed the expansion of oak (*Quercus*) savannas in the Midwest (Abrams 1992), it is unlikely that *I. bullockii* and *I. galbula* came into contact more than 6,500 years ago. In the absence of selection against hybridization, a cline width of 328 km that formed 6,500 years ago would require an
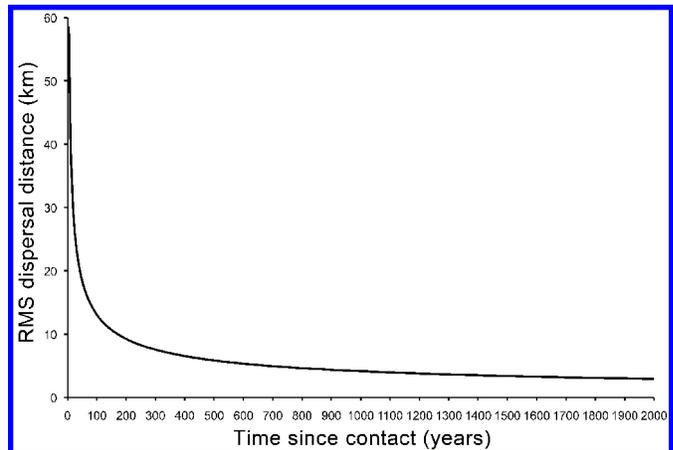

Fig. 4. Relationship between root mean square (RMS) dispersal distance and time since contact ($w = 2.51\sigma\sqrt{t}$), in the absence of selection, if cline width is constrained to the width of the mitochondrial cline (328 km).

RMS dispersal distance of 1.6 km year^{-1}. Estimates of RMS dispersal distances for two other icterids, Common Grackles (*Quiscalus quiscula*) and Red-winged Blackbirds (*Agelaius phoeniceus*), are ~111 km year^{-1} and ~95 km year^{-1}, respectively (Moore and Dolbeer 1989). If we assume ~6,500 years since contact and an RMS dispersal distance of 103 km year^{-1} (the mean of the RMS dispersal estimates for *Q. quiscula* and *A. phoeniceus*), neutral diffusion predicts a cline width of ~21,000 km. A second neutral possibility is that *I. bullockii* and *I. galbula* only came into contact and began hybridizing very recently. If, for example, secondary contact began 100 years before these individuals were collected, we would expect the cline to be ~2,600 km wide (assuming σ = 103 km), about 8× as wide as the observed mitochondrial cline. While at present we can only hypothesize about the RMS dispersal distances of *I. bullockii* and *I. galbula*, to produce the observed cline width (328 km) in the absence of selection would require either very recent contact (~10 to 20 years before sampling) or short RMS dispersal distances (<3.4 km year^{-1} if contact began ≥1,500 years ago; Fig. 4), or both.

Our estimates of cline shape parameters support a role for selection against hybridization in maintaining the width of the hybrid zone between *I. bullockii* and *I. galbula*, at least as it existed ~50 years ago. The selective pressure could be mediated through differences in the scheduling of molt and migration between the species that cause difficulties in hybrid individuals and result in lowered fitness (Rohwer and Manning 1990). A second possibility, which is not mutually exclusive, is that differences in thermoregulatory capabilities of the parental species result in lowered fitness of hybrid individuals outside of the intermediate environmental conditions found in the center of the hybrid zone (Rising 1969, 1983a). Regardless of the mechanism of selection, it is likely that even though mating within the hybrid zone is random (Rising 1983a, b), some selective force is preventing the fusion of *I. bullockii* and *I. galbula*.

Corbin et al. (1979) previously investigated clinal variation between these orioles at allozyme loci and reported very broad clines for the two loci (of 19 total) that were not monomorphic between the species. They did not specifically estimate cline widths, but for both polymorphic loci the greatest transition in allele frequencies

occurred between Ft. Collins, Colorado, and Columbia, Missouri (Corbin et al. 1979), which is a distance of ~1,100 km. Qualitatively, the narrower width of the female-line-specific mitochondrial cline compared with the biparentally inherited autosomal allozyme clines might suggest that female hybrids are less fit than male hybrids, the pattern predicted by Haldane's rule (Haldane 1922). This possibility is consistent with a growing body of evidence that among pairs of avian taxa that hybridize in the Great Plains of North America, male hybrids are usually more fit than their female counterparts, a pattern manifested through reduced introgression and gene flow of mitochondrial loci when compared with autosomal or sex-linked loci (Carling and Brumfield 2008, 2009; Mettler and Spellman 2009; Carling et al. 2010). However, another possibility is that differences in mutation rate and effective population size between mitochondrial and nuclear loci are responsible for the apparent differences in patterns of introgression between these marker classes even if the fitness of males and female hybrids is the same.

Finally, we stress that this study would have been impossible without the exemplary series of specimens collected from across the *bullockii*–*galbula* hybrid zone by Sibley and Short (1964). Increasingly, modern studies of hybrid zones fail to preserve series of voucher specimens that could provide similar resources to future investigators. At times, the ongoing behaviors (such as mate choice and habitat selection) or the future reproductive success of sampled individuals are central components of integrative hybrid-zone studies, making it particularly valuable to collect specimens when the focus is instead on genetic or other characters measured from birds that are not then followed over time in the field.

Literature Cited

Adams, M. 1992. Fire and the development of oak forests. BioScience 42:346–353.

Arnold, M. L. 1997. Natural Hybridization and Evolution. Oxford University Press, New York.

Barton, N. H., and K. S. Gale. 1993. Genetic analysis of hybrid zones. Pages 13–45 in Hybrid Zones and the Evolutionary Process (R. G. Harrison, Ed.). Oxford University Press, New York.

Brelsford, A., and D. E. Irwin. 2009. Incipient speciation despite little assortative mating: The Yellow-rumped Warbler hybrid zone. Evolution 63:3050–3060.

Brumfield, R. T., R. W. Jernigan, D. B. McDonald, and M. J. Braun. 2001. Evolutionary implications of divergent clines in an avian (*Manacus*: Aves) hybrid zone. Evolution 55:2070–2087.

Carling, M. D., and R. T. Brumfield. 2008. Haldane's rule in an avian system: Using cline theory and divergence population genetics to test for differential introgression of mitochondrial, autosomal and sex-linked loci across the *Passerina* bunting hybrid zone. Evolution 62:2600–2615.

Carling, M. D., and R. T. Brumfield. 2009. Speciation in *Passerina* buntings: Introgression patterns of sex-linked loci identify a candidate gene region for reproductive isolation. Molecular Ecology 18:834–847.

Carling, M. D., I. J. Lovette, and R. T. Brumfield. 2010. Historical divergence and gene flow: Coalescent analyses of mitochondrial, autosomal and sex-linked loci in *Passerina* buntings. Evolution 64:1762–1772.

Corbin, K. W., C. G. Sibley, and A. Ferguson. 1979. Genic changes associated with the establishment of sympatry in orioles of the genus *Icterus*. Evolution 33:624–633.

Drummond, A. J., B. Ashton, M. Cheung, J. Heled, M. Kearse, R. Moir, S. Stones-Havas, T. Thierer, and A. Wilson. 2009. GENEIOUS, version 4.8. [Online.] Available at www.geneious.com.

Edwards, A. W. F. 1972. Likelihood: An Account of the Statistical Concept of Likelihood and Its Application to Scientific Inference. Cambridge University Press, Cambridge, United Kingdom.

Endler, J. A. 1977. Geographic Variation, Speciation, and Clines. Princeton University Press, Princeton, New Jersey.

Haldane, J. B. S. 1922. Sex ratio and unisexual sterility in animal hybrids. Journal of Genetics 12:101–109.

Haldane, J. B. S. 1948. The theory of a cline. Journal of Genetics 48:277–284.

Harrison, R. G. 1990. Hybrid zones: Windows on evolutionary process. Pages 69–128 in Oxford Surveys in Evolutionary Biology (D. Futuyma and J. Antonovics, Eds.). Oxford University Press, Oxford, United Kingdom.

Hewitt, G. M. 1988. Hybrid zones—Natural laboratories for evolutionary studies. Trends in Ecology & Evolution 3:158–167.

Irwin, D. E., A. Brelsford, D. P. L. Toews, C. MacDonald, and M. Phinney. 2009. Extensive hybridization in a contact zone between MacGillivray's Warblers *Oporornis tolmiei* and Mourning Warblers and *O. philadelphia* detected using molecular and morphological analyses. Journal of Avian Biology 40:539–552.

Jacobsen, F., N. R. Friedman, and K. E. Omland. 2010. Congruence between nuclear and mitochondrial DNA: Combination of multiple nuclear introns resolves a well-supported phylogeny of New World orioles (*Icterus*). Molecular Phylogenetics and Evolution 56:419–427.

Jiggins, C. D., and J. Mallet. 2000. Bimodal hybrid zones and speciation. Trends in Ecology & Evolution 15:250–255.

Mallet, J. 2005. Hybridization as an invasion of the genome. Trends in Ecology & Evolution 20:229–237.

Mallet, J., and N. Barton. 1989. Inference from clines stabilized by frequency-dependent selection. Genetics 122:967–976.

Mallet, J., N. Barton, G. Lamas, J. Santisteban, M. Muedas, and H. Eeley. 1990. Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *Heliconius* hybrid zones. Genetics 124:921–936.

Mettler, R. D., and G. M. Spellman. 2009. A hybrid zone revisited: Molecular and morphological analysis of the maintenance, movement, and evolution of a Great Plains avian (Cardinalidae: *Pheucticus*) hybrid zone. Molecular Ecology 18:3256–3267.

Moore, W. S., and R. A. Dolbeer. 1989. The use of banding recovery data to estimate dispersal rates and gene flow in avian species: Case studies in the Red-winged Blackbird and Common Grackle. Condor 91:242–253.

NatureServe. 2006. NatureServe Explorer: An Online Encyclopedia of Life, version 6.1. [Online.] Available at www.natureserve.org/explorer.

Omland, K. E., S. M. Lanyon, and S. J. Fritz. 1999. A molecular phylogeny of the New World orioles (*Icterus*): The importance of dense taxon sampling. Molecular Phylogenetics and Evolution 12:224–239.

Porter, A. H., R. Wenger, H. Geiger, A. Scholl, and A. M. Shapiro. 1997. The *Pontia daplidice-edusa* hybrid zone in northwestern Italy. Evolution 51:1561–1573.

Remington, C. L. 1968. Suture-zones of hybrid interaction between recently joined biotas. Pages 321–428 *in* Evolutionary Biology, vol. 2 (T. Dobzhansky, M. K. Hecht, and W. C. Steere, Eds.). Appleton Century Crofts, New York.

Ridgely, R. S., T. F. Allnutt, T. Brooks, D. K. McNicol, D. W. Mehlman, B. E. Young, and J. R. Zook. 2003. Digital distribution maps of the birds of the Western Hemisphere, version 1.0. (CD-ROM.) NatureServe, Arlington, Virginia.

Rieseberg, L. H., and S. E. Carney. 1998. Plant hybridization. New Phytologist 140:599–624.

Rising, J. D. 1969. A comparison of metabolism and evaporative water loss of Baltimore and Bullock orioles. Comparative Biochemistry and Physiology 31:915–925.

Rising, J. D. 1970. Morphological variation and evolution in some North American orioles. Systematic Zoology 19:315–351.

Rising, J. D. 1983a. The Great Plains hybrid zones. Pages 131–157 *in* Current Ornithology, vol. 1 (R. F. Johnston, Ed.). Plenum Press, New York.

Rising, J. D. 1983b. The progress of oriole hybridization in Kansas. Auk 100:885–897.

Rising, J. D. 1996. The stability of the oriole hybrid zone in western Kansas. Condor 98:658–663.

Rising, J. D., and N. J. Flood. 1998. Baltimore Oriole (*Icterus galbula*). *In* The Birds of North America Online (A. Poole, Ed.). Cornell Lab of Ornithology, Ithaca, New York. Available at bna.birds.cornell.edu/bna/species/384.

Rising, J. D., and P. L. Williams. 1999. Bullock's Oriole (*Icterus bullockii*). *In* The Birds of North America Online (A. Poole, Ed.). Cornell Lab of Ornithology, Ithaca, New York. Available at bna.birds.cornell.edu/bna/species/416.

Rohwer, S., E. Bermingham, and C. Wood. 2001. Plumage and mitochondrial DNA haplotype variation across a moving hybrid zone. Evolution 55:405–422.

Rohwer, S., and J. Manning. 1990. Differences in timing and number of molts for Baltimore and Bullock's orioles: Implications to hybrid fitness and theories of delayed plumage maturation. Condor 92:125–140.

Sibley, C. G., and L. L. Short, Jr. 1964. Hybridization in the orioles of the Great Plains. Condor 66:130–150.

Szymura, J. M., and N. H. Barton. 1986. Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and *Bombina variegata*, near Cracow in southern Poland. Evolution 40:1141–1159.

Szymura, J. M., and N. H. Barton. 1991. The genetic structure of the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegate*—Comparisons between transects and between loci. Evolution 45:237–261.

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