



ASSESSMENT OF SPECIES LIMITS AMONG YELLOW-BREASTED MEADOWLARKS (*STURNELLA* SPP.) USING MITOCHONDRIAL AND SEX-LINKED MARKERS

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ABSTRACT.—The yellow-breasted meadowlarks have endured more than a century of debate over species limits and patterns of diversity, ending with the current recognition of two species, the Eastern and Western meadowlarks (*Sturnella magna* and *S. neglecta*), by most authorities. Recently, it has been suggested that a form of the Eastern Meadowlark from the desert Southwest of the United States and northern Mexico is a distinct taxon, Lilian's Meadowlark (*S. lilianae*). We gathered sequence data from the mitochondrial genes cytochrome *b* and ND2 and from an intron of the sex-linked gene aconitase 1, from samples of all three forms from across their ranges. By genealogical concordance, these data strongly support the existence of three differentiated, historically isolated lineages. We recommend species recognition for *S. lilianae* (including the Mexican form *auropectoralis*), and note that its distributional limits in Mexico require clarification. Finally, we note that Z-linked nuclear markers may be particularly useful for lineage delimitation and phylogeny estimation in groups characterized by large variance in male reproductive success. Received 7 September 2007, accepted 21 March 2008.

Key words: grassland birds, North America, species limits, *Sturnella*, systematics, taxonomy, yellow-breasted meadowlarks.

Evaluación de los Límites entre Especies de *Sturnella* Utilizando Marcadores Mitocondriales y Ligados al Sexo

RESUMEN.—Los ictéridos del género *Sturnella* han sido objeto de debates a lo largo de más de un siglo en cuanto a los límites entre especies y a los patrones de diversidad. Esto ha terminado con el reconocimiento actual de dos especies (*S. magna* y *S. neglecta*) por parte de la mayoría de las autoridades. Recientemente, se ha sugerido que una forma de *S. magna* que habita los desiertos del suroeste de los Estados Unidos y el norte de México es en realidad un taxón distinto, *S. lilianae*. Obtuvimos secuencias de los genes mitocondriales citocromo *b* y ND2 y de un intrón del gen ligado al sexo aconitasa 1 para muestras de las tres formas a través de sus rangos. Por concordancia genealógica, esos datos apoyan fuertemente la existencia de tres linajes diferenciados e históricamente aislados, para los cuales recomendamos el reconocimiento a nivel de especie. Recomendamos el reconocimiento de *S. lilianae* (incluyendo a la forma mexicana *auropectoralis*), y notamos que los límites de su distribución en México deben ser aclarados. Finalmente, sugerimos que los marcadores nucleares ligados al cromosoma Z podrían ser particularmente útiles para la delimitación de linajes y la estimación de filogenias en grupos caracterizados por una alta varianza en el éxito reproductivo entre machos.

CURRENTLY, TAXONOMY RECOGNIZES two species of yellow-breasted meadowlarks: the Eastern Meadowlark (*Sturnella magna*) and Western Meadowlark (*S. neglecta*). The Eastern Meadowlark was first figured by Catesby in his *Natural History of Carolina, Florida, and the Bahama Islands* (Feduccia 1985), in which it was seen by Linnaeus, who named it *Alauda magna* in the 10th edition of *Systema Naturae* (von Linné 1956), in honor of Catesby's colloquial nomen "Large Lark." Subsequently, Linnaeus moved the meadowlark to *Sturnus*, and eventually it was placed in *Sturnella* by Vieillot (Vieillot 1816). Nearly a century after the Eastern Meadowlark was described, John James Audubon heard the unusual songs of meadowlarks

near Fort Croghan (Council Bluffs, Iowa). After later examining specimens from the same region, he named the Missouri Meadowlark (now Western Meadowlark), *Sturnella neglecta* (Audubon 1967), acknowledging that the form was first reported by Lewis and Clark in their published journals. The specific status of the latter form remained controversial for more than another century, with early authorities (Allen 1871, American Ornithologists' Union 1895) considering it a subspecies of the Eastern Meadowlark and, later, a full species (Chapman 1900, Ridgway 1902, Hellmayr 1937). Seminal behavioral and morphometric work (Lanyon 1956, 1957, 1966; Rohwer 1972, 1973, 1976), coupled with mating experiments that

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demonstrated hybrid sterility (Lanyon 1979), firmly established the species status of the Western Meadowlark under evolutionary, phylogenetic, and biological species criteria (Mayden 1997).

Only one other form of yellow-breasted meadowlark has been proposed for recognition at the species level. Lilian's Meadowlark, a pale form from the desert grasslands of the southwestern United States and northern Mexico, was described as a subspecies of *Sturnella magna* by Oberholser (1930). Using linear discriminant methods, Rohwer (1976) demonstrated that this taxon was morphologically as distinct from other forms of *S. magna* as both were from *S. neglecta*, but he made no formal proposal for its recognition. Subsequent workers (Sibley and Monroe 1990, Lanyon 1995, Jaramillo and Burke 1999) all noted work by Jon C. Barlow (unpubl. data) that suggested genetic and song differentiation of Lilian's Meadowlark. More recently, Cassell (2002) found significant differences in the songs of Eastern and Lilian's meadowlarks. However, given the learned nature of song in this group (Lanyon 1957), and the sharing of unlearned call notes between these forms (Lanyon 1957, 1962), the relevance of this observation with regard to species limits remains an open question. Here, we analyze molecular data from meadowlarks to determine the number of distinct evolutionary lineages in this clade of grassland birds.

METHODS

Taxon and population sampling.—We sampled across the range of *S. magna*, including North, Central, and South America and an isolated population on Cuba (Table 1 and Fig. 1), representing all disjunct populations and 12 of 14 recognized subspecies (Blake 1968). In particular, we included an appreciable sample of Lilian's Meadowlarks from Arizona, New Mexico, and northern Mexico. Individuals of *S. neglecta* were chosen to sample the extremes of its range (Fig. 1). Several individuals from Florida and the Gulf coast of Texas were taken during the fall or winter and may represent migrant *S. m. magna* rather than *S. m. argutula* (Table 1); however, two winter individuals from Brownsville were identified as the local *S. m. hoopesi* by plumage (R. W. Dickerman pers. comm.). We included the red-breasted meadowlarks *Leistes militaris* and *S. bellicosa*, as well as the more distant relative *Dolichonyx oryzivora* (Lanyon and Omland 1999) as outgroups for our analysis.

Character sampling.—We sampled mitochondrial DNA (mtDNA) sequences from all individuals, including both cytochrome *b* and NADH dehydrogenase subunit 2 (ND2) from all individuals for which we could obtain frozen tissue samples, and cytochrome *b* alone for individuals sampled as dried skins. Because we were particularly interested in defining species limits under a lineage-species concept (Wiley 1978, de Quieroz 1998), and the use of single-marker systems in species delimitation has important limitations (Funk and Omland 2003, Weisrock et al. 2006), we also examined variation at a single nuclear-encoded locus. We selected a Z-linked marker, which has a theoretically lower effective population size than autosomal markers and should, therefore, be more sensitive to population substructuring. Specifically, we obtained sequences from the ninth intron of the nuclear-encoded aconitase 1 gene (ACO1-19). This gene is sex-linked in *Gallus* (Schmid et al. 2000), and allozyme data suggest that its sex linkage is widespread in birds (Baverstock et al. 1982).

Laboratory methods.—DNA was extracted from both skins and fresh tissue samples using the DNeasy Tissue Kit (Qiagen,

Valencia, California). All skin extractions and polymerase chain reaction (PCR) setups were conducted in a separate building where potential avian contaminants were unlikely. For tissue samples, a 1,207-base pair (bp) piece of mitochondrial DNA, including the complete cytochrome-*b* gene (MT-CYTB) and partial sequences of the flanking genes, was amplified by PCR in three pieces (~500 bp each; primer pairs L14857/H15298, L15191/H15709, L15656/H16065; Table 2). This same region was amplified from skin samples in five pieces (~300 bp each; L14857/H15103, L15068/H15460, L15410/H15709, L15656/H15934, L15848/H16065 or L15848/H16137; Table 2). A 1,087-bp piece of NADH dehydrogenase subunit 2 (MT-ND2) and flanking sequence was amplified for tissue samples only using primer pair L5215/H6297 or L5209/H6434 (Table 2). These products were either sequenced directly using the end primers and two internal primers (L5758emb and H5766emb) or reamplified and sequenced in two pieces with end primers (~540 bp each; primer pairs L5215/H5766emb, L5758emb/H6297; Table 2). The sex-linked ACO1-19 was amplified from a subset of individuals for which we had frozen tissue samples. Primary genomic amplifications targeted two fragments (primer pairs: ACO1-19F/ACO1-19IntR, ACO1-19IntF/ACO1-19R; Table 2). Because we were interested in identifying fixed differences between species, if any, we made no attempt to resolve the phase of diploid genotypes in males. All PCR amplifications were performed using a hot start procedure (HotStarTaq, Qiagen) with an initial denaturation at 95°C (15 min), 35–40 cycles of denaturation at 95°C (30 s), annealing at 52–58°C (30 s), extension at 72°C (45 s), and a final extension at 72°C (3 min).

The PCR products were prepared for sequencing by enzymatic digestion with a mixture of exonuclease 1 and shrimp alkaline phosphatase (USB, Cleveland, Ohio), following Werle et al. (1994). Cycle-sequencing was performed using BigDye Terminator, version 3.1 chemistry (following manufacturer's recommendations), and reactions were run on an ABI 3700 automated sequencer. Sequences were edited and aligned by hand using SEQUENCHER (GeneCodes, Ann Arbor, Michigan). Potential heterozygous sites in genotypes sequenced from the nuclear locus were identified by visual inspection of quality values from aligned reads.

Phylogenetic analyses.—We analyzed three data sets with progressively restricted taxon sampling, and a fourth comprehensive data set. These included (1) all unique haplotypes of cytochrome *b* (including sequences derived from skin); (2) all unique haplotypes derived by combination of cytochrome *b* and ND2 sequences (only individuals with fresh tissue); (3) all ACO1-19 genotypes from a subset of individuals included in the second data set; and (4) cytochrome *b*, ND2, and ACO1-19 sequences from all individuals representing unique mitochondrial haplotypes, and a proportion of missing data. Each of the first three data sets was analyzed using equally weighted parsimony (MP), maximum likelihood (ML) (both using PAUP*, version 4.0b10; Swofford 2000), and Bayesian inference (MRBAYES, version 3.0b4; Ronquist and Huelsenbeck 2003), and the fourth was analyzed using Bayesian methods alone. Models for ML and Bayesian analysis were chosen using Akaike's information criterion (AIC), as calculated using MODELTEST, version 3.06 (Posada and Crandall 1998), with parameters estimated by PAUP* on an arbitrarily selected MP tree for each data set. This analysis was supplemented with explicit tests of clock-like behavior for each partition separately, based on the likelihood ratio (Felsenstein 1981). The Bayesian analyses of concatenated

TABLE 1. Taxonomic designations, collection localities, and specimen data for individuals included in the present study. Because of the migratory behavior of some North American *Sturnella magna* and *S. neglecta*, months of collection are given, where known, for areas where migrants occur. Museum samples highlighted by a dagger indicate that DNA was extracted from the toe pad of a round skin.

Taxon	Locality	Museum number ^a	Month ^b	Genotypes ^c
<i>Sturnella magna magna</i>	USA: Minnesota, Olmsted County, Rochester	FMNH 356947	May	cNA1, mtNA1.1, acoNA1
	USA: Minnesota, Ramsey County, Mounds View Long Lake Road	MMNH [X8743]	October	cNA2, mtNA2
	USA: Illinois, Cook County, Field Museum	FMNH 364085	April	cNA1, mtNA1.0, acoNA1
	USA: Illinois, Cook County, McCormick Place	FMNH 333793 FMNH 428478	October March	cNA3, mtNA3, acoNA3 cNA4, mtNA4, acoNA4
<i>S. m. argutula</i>	USA: Wisconsin, Price County	FMNH 428990	?	cNA5, mtNA5, acoNA1
	USA: Florida, Collier County, CR 946, 7 miles east of CR 849	FMNH 393588	May	cNA6, mtNA6, acoNA1
	USA: Florida, Highlands County, Archbold Biological Station	FMNH 432675	May	cNA7, mtNA7, acoNA1
	USA: Florida, Highlands County, Avon Park Air Force Range	FMNH 393589	February	cNA1, mtNA1.3, acoNA1
	USA: Florida, Highlands County, Avon Park Air Force Range	FMNH 428717	February	cNA1, mtNA1.5, acoNA1
		FMNH 432674	April	cNA1, mtNA1.0, acoNA2
	USA: Florida, Highlands County, Buck Island Ranch	FMNH 387734 FMNH 396894 FMNH 393590	October February May	cNA1, mtNA1.7, acoNA1 cNA8, mtNA8, acoNA1 cNA1, mtNA1.4, acoNA1
	USA: Florida, Highlands County, Lake Placid, Jefferson Avenue	MSB NK116442	August	cNA9, mtNA9
	USA: Texas, Jefferson County, Winie, 6 miles east, 4 miles south	MSB NK103192	December	cNA1, mtNA1.2
	USA: Texas, Chambers County, Anahuac, 3 miles south, 4 miles east	MSB NK37162	April	cNA10, mtNA10
	USA: Texas, Matagorda County, 1 mile south of Wadsworth	MSB NK8868	December	cNA11, mtNA11
<i>S. m. hoopesi</i>	USA: Texas, Calhoun County	TCWC [KAA 7087]	May	cNA12, mtNA12
	USA: Texas, Cameron County, 12 miles east of Brownsville	MSB NK37172	December	cNA1, mtNA1.0
		MSB NK37174	December	cNA1, mtNA1.6
<i>S. m. mexicana</i>	Mexico: Veracruz, Tuxtla Mountains	MMNH 35021†	April	cNA13, mtNA13
<i>S. m. griscomi</i>	Mexico: Yucatán; Progreso	MMNH 25287†	November	cNA14, mtNA14
<i>S. m. alticola</i>	Mexico: Chiapas, La Trinitaria	MMNH 25257†	January	cNA15, mtNA15
<i>S. m. subulata</i>	Panama: Panama, Cerro Campana	UBC [JTW 326]	Resident	cMA1, mtMA1.0
	Panama: Cocle, Penenome	UBC [JTW 367]	Resident	cMA1, mtMA1.0
	Panama: Cocle, Rosario	UBC [JTW 578]	Resident	cMA1, mtMA1.1
<i>S. m. meridionalis</i>	Venezuela: Mérida, Pueblo Llano	AMNH 825222†	Resident	cSA1
<i>S. m. paralius</i>	Venezuela: Falcón, Yaracal, 2 km northeast	FMNH 339779	Resident	cSA2, mtSA2, acoNA1
		FMNH 339780	Resident	cSA3, mtSA3, acoNA1
<i>S. m. monticola</i>	Brazil: Roraima, Fazenda Tres Estrelas, 26 km east-northeast of Boa Vista	FMNH [DFS92–248]	Resident	cSA4, mtSA4, acoNA1
<i>S. m. quinta</i>	Brazil: Amapá; Fazenda Itapoã	FMNH 391647	Resident	cSA5, mtSA5, acoNA1
<i>S. m. hippocrepis</i>	Cuba: La Habana, Caimito	AMNH 832019†	Resident	cCU1
		AMNH 832020†	Resident	cCU2
<i>S. m. lilianae</i>	USA: Arizona, Cochise County, Palominas, 2.5 miles south, 5 miles west	MSB NK8864	?	cLI1, mtLI1.3, acoLI1
	USA: Arizona, Santa Cruz County, ~5 miles east-northeast of Sonoita	MSB NK103437	March	cLI1, mtLI1.0
	USA: New Mexico, Bernalillo County, northeast of Albuquerque	MSB NK43358	July	cLI1, mtLI1.1
	USA: New Mexico, Quay County, Nara Visa, 5 miles north	MSB NK37161	?	cLI1, mtLI1.0
	USA: New Mexico, Quay County, 8 miles north, 3 miles east of Logan	MSB NK8892	March	cLI2, mtLI2
	USA: New Mexico, Dona Ana County, 1 mile east of county line on Demning Hekk Road	MSB NK103436	March	cLI3, mtLI3

(Continued)

TABLE 1. Continued.

Taxon	Locality	Museum number ^a	Month ^b	Genotypes ^c
<i>S. m. auropectoralis</i>	USA: New Mexico, Lea County, Lovington, 13 miles east, 1 mile south	MSB NK37151	?	cLI1, mtLI1.0, acoLI3
	USA: Texas, Jeff Davis County, Davis Mountains	MSB NK37169	December	cLI1, mtLI1.0
	México: Sonora, Cananea	FMNH 393903	February	cLI1, mtLI1.2, acoLI2
	México: Nayarit, Compostela	MMNH 25264 [†]	January	cAU1
<i>S. neglecta</i>	USA: California, Tehama County, Red Bluff	MMNH 25266 [†]	January	cAU2
	USA: Texas, Hartley County, Romero, 9.3 miles east	AMNH [JJW 088]	May	cNE3, mtNE3
	USA: Texas, Hartley County, Channing, 28 miles west	MSB NK5937	December	cNE1, mtNE1.3
	USA: Texas, Hartley County, Channing, 28 miles west	MSB NK37160	January	cNE4, mtNE4,
	USA: Oregon, Jefferson County, 12 miles north of Redmond by road west of Highway 97	MMNH [JK 95087]	June	cNE1, mtNE1.2, acoNE1
	USA: Montana, Liberty County, 5 miles west, 27 miles north of Chester	MMNH [JK 95088]	June	cNE5, mtNE5, acoNE2
<i>S. bellicosa</i>	USA: California, Monterey County, 12 miles north, 16 miles west of Jolon	MMNH [JK 9471]	June	cNE1, mtNE1.0, acoNE1
	Peru	FMNH 341966	June	cNE1, mtNE1.1
	Peru	FMNH 341967	June	cNE2, mtNE2, acoNE3
<i>Leistes militaris</i>	Bolivia: Santa Cruz, 5 km south of Santa Cruz	ANSP 178.118		
<i>Dolichonyx oryzivorus</i>	Bolivia: Santa Cruz, Purubi, 30 km south of San Jose de Chiquitos	FMNH 334657		
	Bolivia: Santa Cruz, Purubi, 30 km south of San Jose de Chiquitos	FMNH 334721		

^aAMNH = American Museum of Natural History, New York; ANSP = Academy of Natural Sciences, Philadelphia; FMNH = Field Museum of Natural History, Chicago; MMNH = James Ford Bell Museum, University of Minnesota, Minneapolis; MSB = Museum of Southwestern Biology, University of New Mexico, Albuquerque; TCWC = Texas Cooperative Wildlife Collection, Texas A&M University, College Station; and UBC = University of British Columbia, Vancouver.

^bMonth of collection.

^cGenotype designations identify all unique haplotypes (mtDNA) or unphased genotypes (nuclear locus), and refer to cytochrome *b* (c), combined cytochrome *b* and ND2 (mt), or ACO1-19 (aco); the two-character designators indicate geographic (NA = North America, MA = Middle America, SA = South America, CU = Cuba) or taxonomic (LI = *liliana*, AU = *auropectoralis*, NE = *neglecta*) origins of given variants, and numbering is arbitrary, with the most frequent haplotype or genotype in a group numbered 1. Haplotypes with identical cytochrome-*b* sequences that are differentiated by variation in ND2 are numbered 1.0, 1.1, etc.

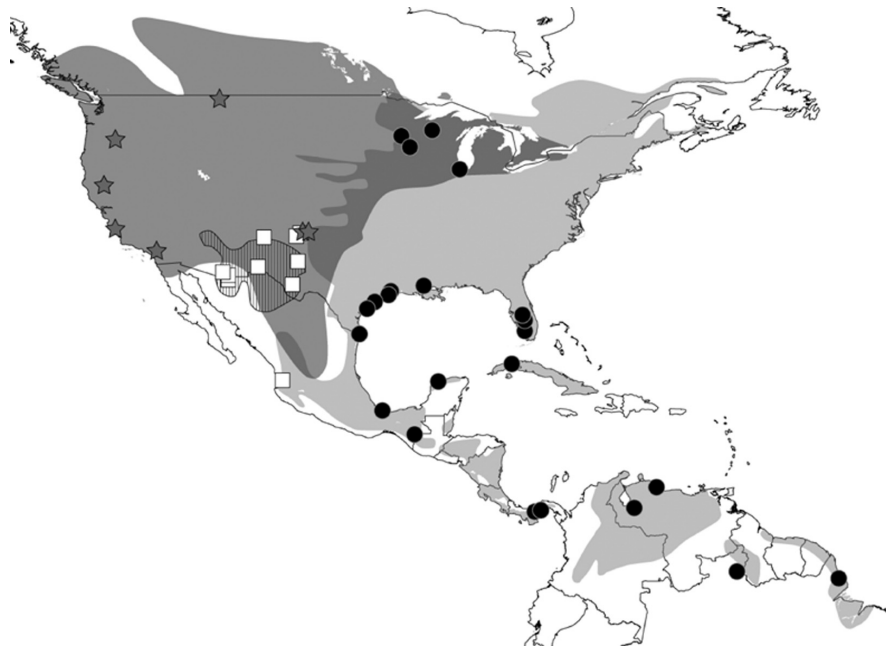


FIG. 1. Map indicating approximate ranges (Ridgely et al. 2003) and sampling of yellow-breasted meadowlark species. Light gray = *Sturnella magna* (sampling localities shown as black circles), dark gray = *S. neglecta* (gray stars), and vertical lines = *S. (magna) liliana* (white squares). The darkest gray shade indicates overlap in the ranges of *S. magna* and *S. neglecta*. The single white square within the range of *S. magna* on the Mexican coast pertains to *S. m. auropectoralis* (see text).

TABLE 2. List of new primers used in amplification and sequencing, numbered according to location in the *Gallus* genome (Desjardins and Morais 1990) for mtDNA loci. Previously published primers used in the present study included (listed by location) L15191 (Lanyon and Hall 1994); L15656, H15298, and H16065 (Helm-Bychowski and Cracraft 1993); H15709 (Barker 2004); H16137 (Sorenson et al. 1999); L5209 and H6434 (O. Haddrath in Bridge et al. 2005); L5215 (Hackett 1996); and H6297 (Drovetski et al. 2004).

Primer	Sequence (5'-3')	Location	Gene	Origin ^a
ND5emb1	AGGATCATTGCGCCTATCCAT	L14857	cyt <i>b</i>	Present study
L15068	CTAGCCATRCCTAYACAGCAGA	L15068	cyt <i>b</i>	1
L15410	TGAGGCGGATTCTCYGTMGACAA	L15410	cyt <i>b</i>	1
L15656.2	AATCTGCTAGGTGACCCAGA	L15656	cyt <i>b</i>	2
L15848	CCAACTACGATCAATRACYTTCCG	L15848	cyt <i>b</i>	1
H15103	TCAGCCGAATTGKACGTCTCGGCA	H15103	cyt <i>b</i>	1
H15460	GTGGACTAGTGTGAGRCCYACGAT	H15460	cyt <i>b</i>	1
H15934	GGCTAGTTGGCCRATGATGATGAA	H15934	cyt <i>b</i>	1
H16191-Pass	TCTCGWGGGGCGATTCCGGC	H16191	cyt <i>b</i>	3
L5758-Emb	GGCTGAATRGGYCTYAACCAAAC	L5758	ND2	4
H5766-Emb	GARGARAAGGCTARGATTTTTTCG	H5766	ND2	Present study
ACO1-I9F	CTGTGGGAATGCTGAGAGATTT		ACO1-19	Present study
ACO1-I9IntF	CCTCTGTGGTAAMCACAAGCA		ACO1-19	Present study
ACO1-I9IntR	GCAGACCCAAACACAAGTTACAA		ACO1-19	Present study
ACO1-I9R	CTGCAGCAAGGCACAACAGT		ACO1-19	Present study

^a1 = modified from Groth (2000), 2 = modified from Helm-Bychowski and Cracraft (1993), 3 = modified from Sorenson et al. (1999), and 4 = modified from Johnson and Sorenson (1998).

genes (data sets 2 and 4) applied the optimal models for each partition, with all parameters independently estimated and branch lengths enforced to proportionality, whereas the ML combined analysis of mtDNA (data set 2) employed uniform parameters and rates. Model parameters estimated on the parsimony trees were fixed during subsequent ML searches. Heuristic searches under MP and ML were performed via tree bisection and reconnection branch-swapping following 50 (MP) or 10 (ML) random sequence additions. Support for these analyses was assessed via the non-parametric bootstrap (1,000 and 200 replicates for MP and ML, respectively; Felsenstein 1985). Bayesian analyses were run using Metropolis coupling with one cold and three heated chains for a total of 2×10^6 generations, with at least two runs per data set initiated from random starting points (Huelsenbeck et al. 2002). The portion of each run prior to stable log-likelihood values was discarded as burn-in. Convergence of runs and stability of estimated nodal posterior probabilities were evaluated using TRACER, version 1.3 (Rambaut and Drummond 2004).

RESULTS

For simplicity, from this point on we will refer to populations of *Sturnella magna*, not including *S. m. lilianae* and *S. m. auropectoralis*, as "Eastern Meadowlark," and to the latter two subspecies as "Lilian's Meadowlark" (see below).

Data and polymorphism.—We obtained full-length cytochrome-*b* sequences from 34 individuals of Eastern, 11 of Lilian's, and 8 of Western meadowlarks (Table 1), with the addition of complete ND2 sequences from a subset of those (28, 9,

and 8, respectively). In addition, we obtained complete sequences of the sex-linked intron 9 of aconitase 1 from 17 Eastern Meadowlarks, including individuals from both North America and South America, as well as from 3 Lilian's and 4 Western meadowlarks. Sequences of these same loci were also obtained for the outgroup taxa, either by sequencing *de novo* or from GenBank (all sequences were obtained from the same individuals). All sequences have been submitted to GenBank (updates to Accessions AF089038 and AF089063; new sequences FJ154607–FJ154732).

Nucleotide diversity (π ; Kimura 1968) at cytochrome *b* ranged from 0.2% in Western and Lilian's meadowlarks to 0.5% across the entire range of Eastern Meadowlark. North American populations of Eastern Meadowlark harbored approximately half the diversity of the group as a whole. The Z-linked locus exhibited significantly lower polymorphism than mtDNA, which is consistent with the lower mutation rate expected for avian nuclear genes (Johnson and Clayton 2000, Prychitko and Moore 2000, Sheldon et al. 2000, Barker 2004). Because we did not isolate alleles, we simply report the numbers of segregating sites: 3 (including a single 1-bp indel) in Eastern, 4 in Lilian's, and 6 (including one 4-bp indel) in Western meadowlarks.

Phylogenetic analyses.—Phylogenetic analysis of the cytochrome-*b* data using MP, ML, and Bayesian methods yielded congruent estimates of haplotype relationship (Fig. 2A and Table 3), all of which strongly supported two monophyletic clades that correspond to plumage coloration (a yellow-breasted and a red-breasted clade), and identified three well-supported haplotype groups within yellow-breasted meadowlarks, corresponding to Western (*S. neglecta*), Lilian's (*S. m. lilianae* and *S. m. auropectoralis*), and Eastern (all other *S. magna*) meadowlarks

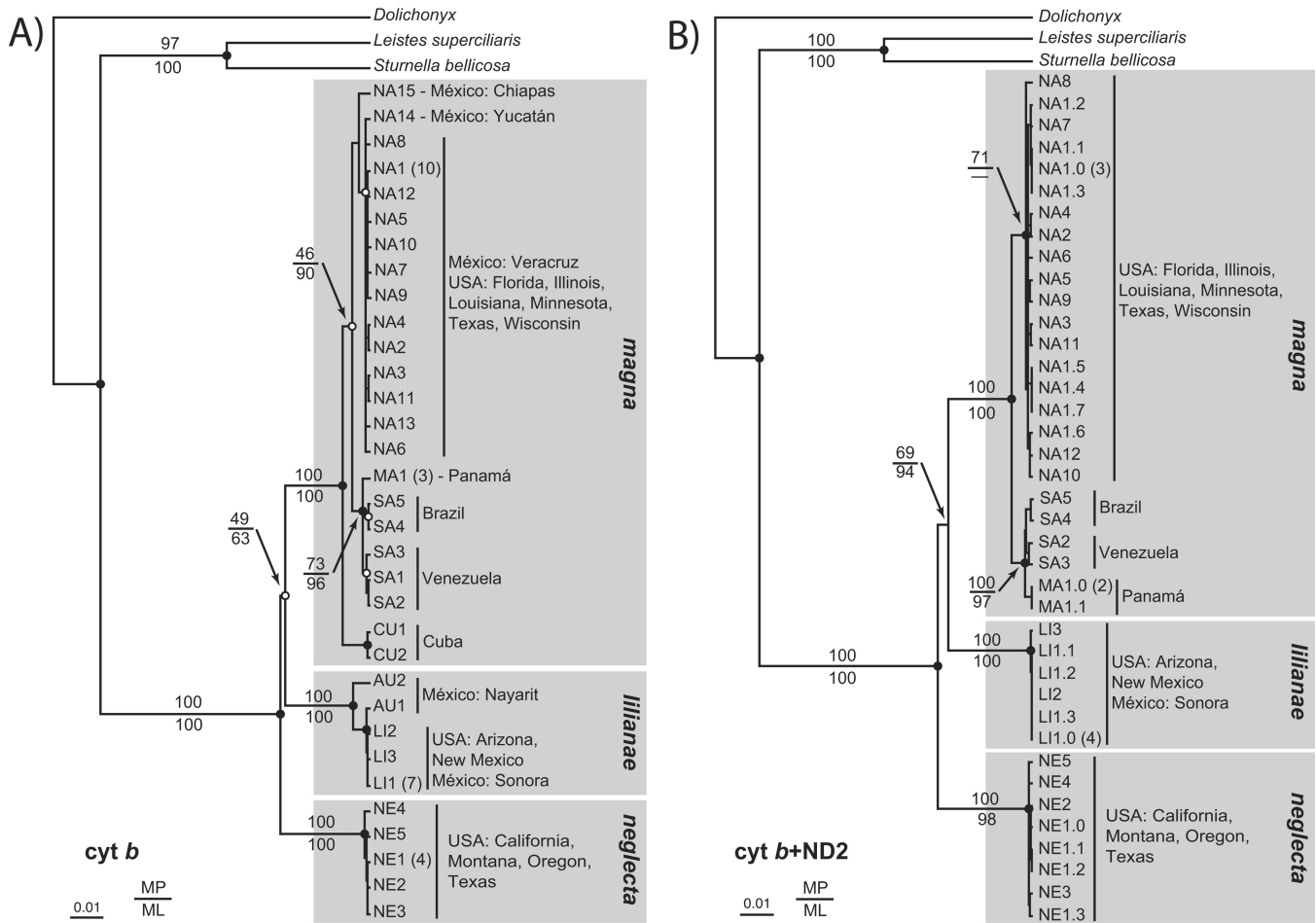


FIG. 2. (A) Phylogenetic analysis of unique cytochrome-*b* haplotypes of yellow-breasted meadowlarks (numbers in parentheses indicate frequencies of occurrence > 1; see Table 1 for assignments to individuals). Shown is the maximum-likelihood (ML) tree of relationships (see Table 3 for parameterization), with branch lengths proportional to expected divergence. The shaded boxes indicate Eastern (*magna*), Lilian's (*lilianae*, *sensu lato*), and Western (*neglecta*) meadowlarks, and localities where given haplotypes were found are indicated. Recovery of given relationships as indicated by nonparametric bootstrapping is shown at each node (parsimony above, likelihood below). All nodes with estimated Bayesian posterior probabilities ≥ 0.95 for the combined mtDNA and nuclear data set are marked by circles: the subset of nodes with estimated probabilities ≥ 0.95 with the cytochrome-*b* data alone are indicated by solid black circles. (B) Phylogenetic analysis of unique cytochrome *b* + ND2 haplotypes of yellow-breasted meadowlarks (see Table 1 for assignments to individuals). Shown is the ML tree of relationships (see Table 3 for parameterization), with branch lengths proportional to expected divergence. Shading is as in Figure 2A. Recovery of given relationships as indicated by nonparametric bootstrapping is shown at each node (parsimony above, likelihood below), and estimated Bayesian posterior probabilities ≥ 0.95 are indicated by closed circles.

(Fig. 2A). All three analyses agreed in placing the Lilian's cluster with the Eastern group, but support for this arrangement was not strong (Fig. 2A). Within the Lilian's cluster, one haplotype of *S. m. auropectoralis* was closely related to haplotypes of *S. m. lilianae*, whereas the second was more divergent ($\sim 1\%$; Fig. 2A). Within the Eastern cluster, more structure was apparent, with two Cuban haplotypes (*S. m. hippocrepis*) sister to the remainder ($2.2 \pm 1.3\%$ [SD] divergent), and a monophyletic cluster of South and Middle American haplotypes sister to a more northerly group ($1 \pm 0.1\%$ divergent; Fig. 2A). Only the South and Middle American clade received notable support (Fig. 2A). Analysis of the combined mtDNA data yielded trees congruent among methods and consistent with those obtained from the cytochrome-*b* data

alone (Fig. 2B and Table 3). The sister relationship between Lilian's and Eastern meadowlarks received higher support in this analysis, as did the South and Middle American clade of Eastern Meadowlarks (Fig. 2B).

Analysis of the Z-linked gene yielded trees congruent with those obtained from mtDNA (Fig. 3). However, the nuclear data strongly supported a sister-group relationship between Lilian's and Eastern meadowlark groups (Fig. 3). Moreover, there were eight fixed differences between these two genotype clusters, and 15–16 fixed differences between them and the Western group. By contrast, the nuclear data failed to recover any significant structure within any of the sampled groups. In particular, the distinction between South–Middle and North American individuals was not

TABLE 3. Results of phylogenetic analyses of haplotypic and genotypic data from yellow-breasted meadowlarks. Data-set sizes, amounts of variation, measures of fit, and maximum-likelihood (ML) model parameterization are given for the two mitochondrial genes independently and in combination, as well as for the nuclear intron. All models included the molecular clock (see text). MP = maximum parsimony, L = parsimony tree length, CI = ensemble consistency index, RI = ensemble retention index, model = best-fit model selected by Akaike's information criterion (see text), and $-\ln(\ell)$ = negative log-likelihood score of ML trees.

	Data sets			
	CYTB	ND2	CYTB+ND2	ACO1-I9
Aligned length	1,208	1,077	2,285	1,002
Number variable	226	273	494	56
Number informative	131	164	292	26
Number of trees (MP)	6	40	290	1
L	323	372	670	58
CI	0.783	0.790	0.806	0.983
RI	0.896	0.916	0.926	0.977
Model	HKY+I+G ₄	GTR+I+G ₄	GTR+G ₄	HKY
Number of trees (ML)	149	>920	183	3
$-\ln(\ell)$	3,379.86	3,165.79	6,397.04	1,756.48

recovered, with individuals from Venezuela and Brazil sharing genotypes with individuals in Minnesota and Illinois (Table 1).

Combined analysis of the data from all genetically uniquely identifiable individuals yielded a strongly supported tree, despite significant missing data (e.g., ND2 and ACO1-I9 from all skin samples, ACO1-I9 from some individuals with tissues). These relationships are not shown, because they are essentially identical to those estimated for cytochrome *b* alone; however, the combined analysis differed in the presence of significant support for a number of important nodes not supported by either cytochrome *b* or the

combined mtDNA data (Fig. 2A). In particular, the combined analysis yielded strong support for a sister-group relationship between the Eastern and Lilian's genotype groups, for monophyly of the continental variation within the Eastern genotype group in relation to the Cuban *S. m. hippocrepis*, and for monophyly of the North American samples within the Eastern genotype group, excepting the sample of *S. m. alticola* from Chiapas, Mexico (all with estimated posteriors = 1.00).

DISCUSSION

Three species of meadowlark in North America.—Our data, obtained from two unlinked genetic loci, agree in delineating three genotypic groups of yellow-breasted meadowlarks. The large divergence among these groups, and the relative lack of differentiation within them, indicates a long history of isolation, consistent with species status under a lineage concept (Wiley 1978, de Quieroz 1998). Taxonomically, the groups correspond to the Western Meadowlark (*S. neglecta*), Eastern Meadowlark (*S. magna* without *liliana*e and *auropectoralis*), and Lilian's Meadowlark (*S. [magna] liliana*e and *S. [m.] auropectoralis*). Currently, we have no nuclear data supporting the union of *S. [m.] liliana*e and *S. [m.] auropectoralis*; however, the mtDNA data are unequivocal on this point and, in the absence of contradictory data, these two forms should be united taxonomically. We propose to recognize this group at the species level, with the name *Sturnella liliana*e, in deference to the description of this form by Oberholser (1930), four years before Saunders (1934) described *S. [m.] auropectoralis*. Recognition of these forms as a species is in agreement with their distinctiveness, as previously recognized by morphology (Rohwer 1976) and song (Cassell 2002).

Recognizing *S. liliana*e has unexpected consequences with regard to interpretation of the distribution of meadowlarks in Mexico. We have demonstrated a clear genetic link between *S. [m.] liliana*e and *S. [m.] auropectoralis*; however, these two forms do not appear to be reciprocally monophyletic with regard to mtDNA data. To date, there has been no quantitative analysis

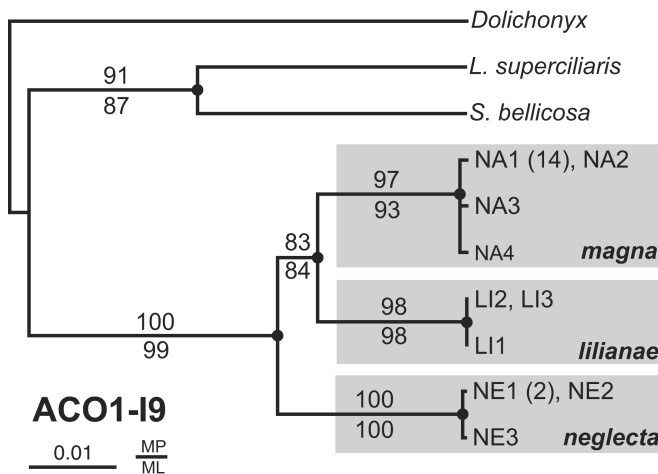


FIG. 3. Phylogenetic analysis of unique ACO1-I9 genotypes of yellow-breasted meadowlarks (numbers in parenthesis indicate frequencies of occurrence >1; see Table 1 for assignments to individuals). Genotypes of *Sturnella magna* were sampled from North, Middle, and South America. Shown is the consensus relationship for all methods of analysis, with branch lengths optimized by maximum likelihood (see Table 3 for parameterization). Shading and support values are as in Figure 2B.

of the morphological differentiation between them, though at the extremes of their range in the desert Southwest of the United States and in the transvolcanic belt of Mexico they appear to be distinct. In the absence of other quantitative studies, we consider the species status of these two forms uncertain, and we propose to give them formal recognition as subspecies (in taxonomies that recognize this rank) within a broadly construed *S. lilianae* that includes both southwestern U.S. and Mexican populations. Extending the definition of *S. lilianae* to include *S. [m.] auropectoralis* begs the question of which Mexican populations pertain to *S. lilianae* and which to *S. magna*; however, it is beyond the scope of the present study to delimit the ranges of these forms exactly. Although multiple lines of evidence suggest complete isolation of *S. lilianae*, *S. magna*, and *S. neglecta* in the United States (Lanyon 1966, 1979; Rohwer 1972, 1976), additional work will be necessary to determine whether introgression between *S. lilianae* and *S. magna*, in particular, may occur via *S. l. auropectoralis* in central Mexico. If found, such introgression might affect the status of *S. lilianae* under a biological, but not a lineage, species concept (Mayden 1997).

Variation within Sturnella magna.—In addition to the discrete genotypic differences between the three yellow-breasted meadowlarks, we noted significant intraspecific variation within *S. magna*. In particular, we found our two samples of *S. m. hippocrepis* to be sister to all continental variation, with strong support from cytochrome-*b* data (Fig. 2A). Our sequences from the Cuban birds are ~2.2% divergent (uncorrected *p*) from the continental haplotypes, which is remarkably similar to reported values between other endemic Cuban forms and their mainland counterparts (Fleischer et al. 2006, Barker et al. 2008). As yet, we do not have sufficient molecular or phenotypic data to argue for species status; however, the Cuban form appears to differ somewhat in body proportions and song (Chapman 1900), and future work may lead to recognition of the Cuban birds at the species level.

The next most significant break within *S. magna* involves the reciprocal monophyly of North American versus Middle and South American forms (Fig. 2). Although this break (~1% divergence) is found in our analyses of mtDNA, no significant differentiation appears at the single nuclear locus we sampled (Fig. 3 and Table 1). The significance of this break is most likely overemphasized by our sampling, which has taken relatively few individuals from distant geographic regions (Irwin 2002). More extensive sampling across Chiapas and Panama (where these haplotype clades likely meet) will further clarify population structure for the species; however, available genetic and morphological data (Dickerman and Phillips 1970, Dickerman 1989) are consistent with recognition of a single polytypic species.

Our data also have significant bearing on subspecific taxonomy of meadowlarks. As previously recognized, the Eastern Meadowlark is the second most polytypic icterid, with 17 subspecies, bested only by the Red-winged Blackbird (*Agelaius phoeniceus*) with 24 (Blake 1968). Our data suggest removal of two of these forms (*lilianae* and *auropectoralis*) into a second species, leaving 15 within *S. magna*. However, the data also suggest that only one of these forms (the Cuban *S. m. hippocrepis*) is substantially differentiated, the other major genetic break being between South and Middle American forms on the one hand and North American (including Mexican) forms on the other. Thus, the pattern of differentiation in this group is consistent with that observed for

many Nearctic and Palearctic bird species (Zink 1996, 2004; Phillimore and Owens 2006). It is likely that the observed plumage variation among populations reflects relatively recent local adaptation rather than a prolonged history of isolation.

Sex-linked loci in delimitation of avian lineages.—Ideally, studies of species limits should incorporate multiple lines of evidence, from morphology, behavior, and unlinked genetic loci (Baum and Shaw 1995, Wiens and Servedio 2000, Wiens and Penkrot 2002). However, it is increasingly common to identify evolutionary units using genetic data alone, with particular reliance on mitochondrial sequence data (Avice et al. 1987, Moritz et al. 1987; see discussion in Funk and Omland 2003). Although mtDNA can be particularly useful for detecting population structure because of its rapid substitution rate and small effective population size (e.g., Wiens and Penkrot 2002), the utility of mtDNA in delimiting lineages and inferring population history has been questioned on a variety of grounds (Edwards and Beerli 2000, Ballard and Whitlock 2004, Moritz and Cicero 2004, Edwards et al. 2005). In particular, mtDNA has been shown to be subject to selection (McDonald and Kreitman 1991, Nachman et al. 1994, Fry and Zink 1998, Ballard and Whitlock 2004; but see Ballard et al. 1996, Barton and Etheridge 2004). Likewise, a single locus can exhibit significant geographic structure under simple isolation by distance, in particular if relatively sparse population samples are analyzed (Irwin 2002). Finally, mtDNA in birds may underestimate gene flow between partially isolated but interbreeding groups, because of the action of Haldane's rule (Orr 1997), which restricts exchange of markers associated with the heterogametic sex (females in birds). However, this constraint may be less important under lineage, as opposed to biological, species concepts. All of these potential biases and limitations emphasize the need for multiple lines of evidence in species delimitation.

Here, we have pursued collection of additional data from an unlinked genetic locus. We recognize, however, that not all nuclear loci are equivalent and that some may be more sensitive to isolation than others. In mammals, where the male is heterogametic, the nonrecombining portion of the Y chromosome provides a male-associated counterpart to the maternal mtDNA, with a similarly small effective population size but, generally, a lower evolutionary rate (Petit et al. 2002). In birds, the Z chromosome is associated with males two-thirds of the time and, thus, its history is influenced by male-specific patterns of gene flow and demography. More importantly, all else being equal, the Z chromosome has three-fourths the effective population size of an autosomal locus and, thus, should be slightly more sensitive to the effects of isolation (and the establishment of independently evolving lineages). Fortunately, all else is not equal. Meadowlarks are characterized by polygynous mating systems (Lanyon 1994, 1995): typically in such systems, the variance in male reproductive success exceeds that in females, sometimes substantially (e.g., by an order of magnitude in Red-winged Blackbirds; Orians and Beletsky 1989). This reproductive skew has the effect of reducing the effective population size of male-associated markers such as the Z chromosome (Nunney 1993, Hedrick and Parker 1997), beyond the expected reduction due to ploidy. Thus, male-associated markers may be particularly effective for distinguishing the history of isolation in avian lineages characterized by polygyny, particularly given that females are generally the dispersing sex (Greenwood and Harvey 1982).

In fact, our observations of mtDNA structure are borne out by the data from the Z-linked ACO1 gene (Fig. 3 and Table 1). These data clearly delineate the same three lineages as the mtDNA, each supported by multiple fixed differences (see above). Observed differentiation between the Eastern Meadowlark and Lilian's Meadowlark is relatively small at ~4% (as assessed at cytochrome *b*), which is nearly the same as the distance between either of those forms and the Western Meadowlark. Despite this small degree of differentiation, we found significant nuclear polymorphism and multiple fixed differences between lineages (e.g., 0.8% divergence between Eastern and Lilian's meadowlarks). This emphasizes another potential advantage of Z-linked loci in birds—male-associated loci, in general, experience elevated rates of evolution (male-driven molecular evolution), which may be caused by increased replication-associated mutation rates (e.g., Kahn and Quinn 1999, Carmichael et al. 2000). Although they are probably not general (but see Peters et al. 2005), we find these results encouraging with regard to our ability to incorporate multiple loci—in particular, sex-linked loci—in lineage identification in birds.

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