GENETIC CONFIRMATION OF THE FIRST DOCUMENTED NESTING BY WESTERN MEADOWLARK (*STURNELLA NEGLECTA*) IN MISSISSIPPI

Sheena Feist[1], Gene C. Knight[2], and Nicholas A. Winstead[1]
[1]Mississippi Museum of Natural Science
2148 Riverside Dr.
Jackson, MS 39202
[2]79 Hwy. 9 W.
Oxford, MS 38655

Abstract

We used molecular techniques to confirm the species identification of a meadowlark nestling (MISS 3167) in the University of Mississippi Vaiden bird collection, housed at the Mississippi Museum of Natural Science. Mitochondrial DNA (mtDNA; Cytochrome B) sequence data obtained from toe pad tissue of MISS 3167 confirms the nestling is a Western Meadowlark (*Sturnella neglecta*). This specimen represents the first documented nesting of Western Meadowlark in Mississippi.

Introduction

Relatively little is known about Western Meadowlarks (*Sturnella neglecta*) in Mississippi. Western Meadowlark was formerly placed on the Mississippi Bird Records Committee's (MBRC) Review Species List due to its rarity within the state. An increase in reported observations led the MBRC to remove the species from that list in the early 2000s. However, the MBRC's most recent Checklist of Birds of Mississippi still labels Western Meadowlark as a rare winter species, meaning it is present singly or in small numbers annually.

Western Meadowlark is found throughout much of western North America, and its nonbreeding range extends into parts of eastern North America more typically occupied by the Eastern Meadowlark (*Sturnella magna*). The two species are similar in appearance, and vocalizations are the best way to distinguish between them in the field. The typical eastern extent of the Western Meadowlark nonbreeding range is not well understood due to difficulty distinguishing between the two species, but the typical eastern extent of its breeding range is from northwest New York, northwest Ohio, north Indiana, central Illinois, northwest Missouri, southeast Kansas, central Oklahoma, west-central Texas, and into Mexico (Davis and Lanyon 2008).

No officially accepted breeding records for Western Meadowlark exist from Mississippi, and few are known from other states outside its typical breeding range (Smith 1951, Lowery 1974). This is in spite of the fact that M.G. Vaiden, arguably Mississippi's most prolific bird collector, reported finding a Western Meadowlark nest in Bolivar County in April 1961 from which he subsequently collected a nestling (MISS 3167, Figure 1; James and James 1961, Vaiden 1961). Vaiden's bird collection, including the meadowlark nestling, was transferred to the University of Mississippi as a teaching and research collection in the late 1960s, and is currently on long-term loan to the Mississippi Museum of Natural Science. It remains a mystery why this record was not accepted by the MBRC or its predecessor at the time, though it could be due to the implausibility of the record and the similarity in appearance of the two species, in addition to there being no good way at the time to distinguish between nestlings of the two species.

The MBRC Chair (GCK) came across the meadowlark nestling while perusing the Vaiden bird collection at the University of Mississippi in late 2015. After some discussion, it was agreed

that the Mississippi Museum of Natural Science should use molecular techniques to confirm the species identity.

Figure 1. MISS 3167, nestling Western Meadowlark, collected from 1961 from Bolivar County, Mississippi by M.G. Vaiden.

Methods

A toe pad was removed from MISS 3167 using sterilized scalpel and tweezers. DNA extraction (using the toe pad) was completed in a clean lab never exposed to amplified DNA, in which the benchtop had been sterilized with 10% bleach. Before extraction began, the toe pad was rinsed in three stages to soften the tissue and remove potential inhibitors (specimen preservatives, e.g., arsenic): 1) 100 µl 100% EtOH for 5 minutes, 2) 100 µl Tris-EDTA buffer for 5 minutes, and 3) 100 µl Polymerase Chain

Reaction (PCR)-grade H_2O for 5 minutes. After rinsing, the toe pad was diced into small pieces with a sterile scalpel, then underwent extraction using Qiagen DNEasy Blood and Tissue Kit with the following modifications: 40 µl Proteinase K was added to the lysis step (instead of 20 µl) with incubation extended to 40 hours; an additional 10 µl Proteinase K was added at the end of incubation; AE buffer was heated to 70°C before use, with incubation extended to 5 minutes; final elution volume was 50 µl twice for a total of 100 µl. A negative extraction was included to detect potential contamination.

A primer set targeting a short but variable region of Cytochrome B (mtDNA) was designed in Primer 3 Plus (Untergasser et al. 2007; Table 1) using a representative sequence obtained from an alignment of multiple GenBank (www.ncbi.nlm.nih.gov/genbank/; Clark et al. 2016) accessioned sequences for both Western Meadowlark (AF290164 and EF529952) and Eastern Meadowlark (AF089063 and FJ154607).

Table 1. Primer set designed to amplify approximately 151 bp of Cytochrome B in both Eastern Meadowlark and Western Meadowlark.

Primer name		Sequence (5' to 3')
LarkCytb151	Forward	TCCACTTTCTCCTCCCCTTT
	Reverse	CCGAGGATGTCTTTGATGGT

PCR reactions were set up in the clean lab in a UV-sterilized hood. PCR reactions were carried out in 25 µl total volumes, using DreamTaq (Thermo Scientific) chemistry and incorporating 10 µl extracted DNA. The PCR profile used a temperature gradient, and occurred in 40 cycles (Table 2). The extraction negative was included in the PCR reaction to test for contamination.

Table 2. Thermalcycler conditions used in conjunction with primer set LarkCytb151 to attempt PCR amplification of MISS 3167 DNA.

Temperature	Duration
95°C	3 minutes
40 cycles:	
95°C	30 seconds
60-54.5°C	30 seconds
72°C	1 minute
72°C	10 minutes

To confirm successful amplification of DNA, PCR products were observed in agarose gel stained with GelRed (Biotium) and illuminated in the presence of ultraviolet light. PCR products from annealing temperatures of 56.5°C and 54.5°C indicated amplification (Figure 2), and thus underwent FastAP (Thermo Scientific) clean-up before undergoing Sanger sequencing at the University of Missouri DNA Core Facility. Resulting chromatograms were visualized, edited, and aligned in Geneious v.7.1.7 (Kearse et al. 2012). Alignment showed that the resulting 151 base-pair sequences were identical at 56.5°C and 54.5°C. Using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Information (National Center for Biotechnology Information 2016), the resulting consensus sequence was queried against highly similar sequences to obtain a species identification.

Results

The obtained Cytochrome B sequence from MISS 3167 is a 100% match to Western Meadowlark, and only a 97% match to Eastern Meadowlark. As indicated in Table 3, five consistent

differences exist within the comparison of MISS 3167 and representative Eastern Meadowlark sequences; zero differences exist between MISS 3167 and representative Western Meadowlark sequences.

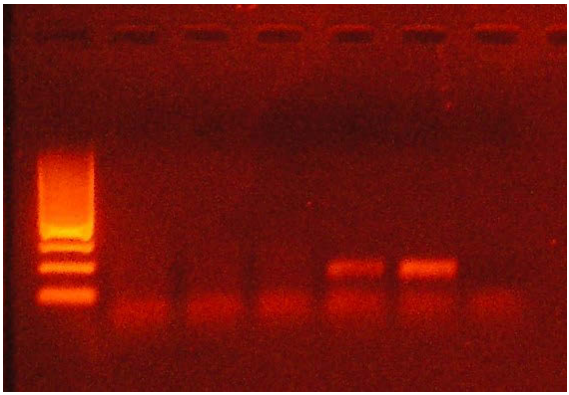

Figure 2. Illuminated agarose gel (stained with GelRed) showing PCR amplification of Cytochrome B at temperatures 56.5°C and 54.5°C for MISS 3167.

Table 3. Variable sites (along 151 base pairs of Cytochrome B) observed between MISS 3167 and representative Eastern (EAME) and Western (WEME) meadowlark sequences. Nucleotides are provided for MISS 3167. Nucleotides identical to MISS 3167 are indicated by •; differences are represented by the observed nucleotide variation.

Sequence	Nucleotide position				
	31	43	46	61	100
MISS 3167	C	C	C	C	T
EAME, Genbank: AF089063	T	T	T	T	C
EAME, Genbank: FJ154607	T	T	T	T	C
WEME, Genbank: AF290164	•	•	•	•	•
WEME, Genbank: EF529952	•	•	•	•	•

Discussion

Our results confirm nesting by Western Meadowlark in Mississippi 56 years ago, and are an example of the importance of properly maintained natural history collections. Without an available specimen and associated tissue, the question of species identity of the nesting birds reported by Vaiden in 1961 might still remain.

Cytochrome B is within the mitochondrial genome which is inherited matrilinealy; therefore, our genetic data can be used to draw conclusions about only the maternal lineage of MISS 3167. However, Vaiden's species determination was based on vocalizations of the birds which he, Mrs. Vaiden, and several protégés observed for over 100 hours between 23 March and mid-May, 1961 (James and James 1961, Vaiden 1961). Vaiden had many years' worth of experience distinguishing between the two meadowlark species, and made multiple collections of both. He verified Western Meadowlarks by their vocalizations prior to collecting them and his specimens were verified by trained ornithologists such as Alexander Wetmore. Eastern and Western meadowlarks are known to hybridize infrequently, but Vaiden's observations combined with the genetic evidence presented here rule out hybridization in this case.

The Vaidens discovered the pair of Western Meadowlarks on 23 March 1961. They observed them for many days before the nest was found with five eggs on 24 April about 3 m from a highway 1.5 km east of Rosedale (James and James 1961, Vaiden 1961). During the second visit on 4 May, the Vaidens found five "very young birds" in the nest. The Vaidens and two protégés visited the nest again on 10 May, when MISS 3167 was collected from the nest, then again on 11 May. The remaining nestlings fledged 12 May.

Interestingly, the Vaidens found at least two additional pairs of Western Meadowlarks near Rosedale on 21 April, and 10 June 1961, respectively (James and James 1961). These observations are suggestive of additional nesting efforts, although there was no indication given of how long into the nesting season each pair was observed and no other nests were discovered. One of the localities where the three pairs were found was regularly used by Western Meadowlarks during the winter, suggesting the nesting birds resided near Rosedale year-round.

Acknowledgments

We thank Dr. Paul Lago for his dedication in maintaining the Vaiden bird collection, and for allowing us access to MISS 3167 while housed at the University of Mississippi. This project was supported by funding through a United States Fish and Wildlife Service State Wildlife Grant.

Literature Cited

Clark, K., I. Karsch-Mizrachi, D.J. Lipman, J. Ostell, and E.W. Sayers. 2016. GenBank. Nucleic Acids Research 44:D67-D72.

Davis, S.K., and W.E. Lanyon. 2008. Western Meadowlark (*Sturnella neglecta*), In The Birds of North America, (P.G. Rodewald, Editor). Cornell Lab of Ornithology, Ithaca, NY. https://birdsna.org/Species-Account/bna/species/wesmea.

James, D., and F.C. James. 1961. Central southern region. Audubon Field Notes 15(2):473-475.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, et al. 2012. Geneious Basic: an integrated and extendable desktop platform for the organization and analysis of sequence data. Bioinformatics 28:1647-1649.

Lowery, G.H., Jr. 1974. Louisiana Birds. Louisiana State University Press, Baton Rouge, LA.

National Center for Biotechnology Information. 2016. Database resources of the National Center for Biotechnology Information. Nucleic Acids Research 44:D7-D19.

Smith, D. 1951. Western Meadowlark nesting at Memphis. The Migrant 22(2):21-22.

Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J.A.M Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Research 35:W71-W74.

Vaiden, M.G. 1961. Some birds of the Yazoo-Mississippi Delta. Mississippi Delta Naturalists' Club Occasional Papers 1(3):1-4.