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DNA FROM FECES AND MUSEUM SPECIMENS CONFIRMS
A FIRST STATE RECORD BIRD

Alec R. Lindsay¹ and Skye C. G. Haas¹

ABSTRACT.— In October 2005, a vagrant kingbird (*Aves: Tyrannus* sp.) appeared in Michigan's Upper Peninsula, nearly 2000 km from the northern limit of its usual range. Using mitochondrial DNA obtained from a fecal sample deposited by the bird and mitochondrial DNA isolated from museum reference specimens, the species identity of this bird was definitively confirmed as a Tropical Kingbird (*T. melancholicus*) rather than a Couch's Kingbird (*T. couchii*). This is the first time DNA evidence has been used to establish a state bird record, and one of the few studies of any type to successfully use avian feces for DNA analysis. Circumstantial evidence indicates that this bird was possibly displaced from its original range by Hurricane Wilma in October, 2005. Identification of vagrant birds is important for studying avian populations, and non-invasive genetic sampling techniques should be considered when traditional means of identification fail to provide definitive evidence of identity.

Key words: fecal DNA, mtDNA, non-invasive genetic sample, PCR inhibition, vagrant species

INTRODUCTION

Vagrant birds (*e.g.*, individuals that occur outside their normal geographic range) are of interest to conservation biologists for many reasons, ranging from the ecologic to the economic. Records of vagrant birds can be indicators of population growth and range expansion (Veit 2000), and the documentation of vagrant birds can provide insights into the biological ramifications of major climatic shifts (Patten and Marantz 1996) and anthropogenic disturbance (Wehtje 2003). Vagrant birds also have impacts on local human economies, as there is a broad and committed community of amateur and professional enthusiasts who will go to great lengths (*e.g.*, Obmascik 2004) to observe vagrant birds.

Valid identification of vagrant birds is important enough to the general public that each state in the U.S.A. has an official rare bird records committee (there are similar review bodies around the world) that is responsible for reviewing detailed documentation for each putative vagrant bird that is recorded in a state. Documentation of the status of all species that occur in each state (breeding, migrating, vagrant, etc.) is typically determined by a set of accepted

¹Department of Biology, Northern Michigan University, Marquette, MI 49855, U.S.A.

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criteria (Dittmann and Lasley 1992). State records committees typically review written, photographic, audio recording or specimen evidence to establish the validity of a state record and then include each record in a centralized database. These databases provide invaluable sources of information that can be used by researchers to examine any number of ecological, environmental, climatological or conservation questions (Patten and Marantz 1996; Shaffer *et al.* 2007).

In late October of 2005, an unusual vagrant bird appeared in Michigan's Upper Peninsula, near the south shores of Lake Superior in Au Train township (Alger County). The bird (photo: Figure 1b) was identified as one of two species with nearly identical plumage characteristics - either a Tropical Kingbird (*Tyrannus melancholicus*) or a Couch's Kingbird (*T. couchii*). The two species are nearly identical in plumage, and subtle morphological differences can only be used to distinguish these two species in the hand (Pyle 1997). Most field ornithologists separate these species in the field only using vocal characteristics (Howell and Webb 1995). The song of *T. melancholicus* is a monotone or slightly rolling series of metallic trills and its call is a sputtering, twitter of sharp metallic notes. *T. couchii* has a song that is more nasal in tone, and its calls are longer and burrier than those of *T. melancholicus* (Howell and Webb 1995, Sibley 2000). The normal breeding ranges of these two species overlap in eastern portions of Central America, where they are both common. Neither species is common north of the US-Mexican border, although both are recorded in the southeastern-most corner of Texas, and a *T. melancholicus* breeding population exists in southeastern Arizona (Figure 1a, c). This vagrant bird, whether of either putative species, was more than 2000 km away from the nearest extent of its normal breeding range when it was recorded in Michigan's Upper Peninsula.

Given tenuous observational data reported for this vagrant kingbird, we extracted and analyzed DNA from a fecal dropping collected from beneath the perched bird. We sequenced mitochondrial DNA from the unknown kingbird and from tissues of museum specimens of *T. couchii* and *T. melancholicus* and used those sequence comparisons to determine the species identity of the unknown kingbird.

METHODS

Field Observation – On 29 October 2005 a yellow-bellied *Tyrannus* flycatcher was observed along the Au Train River near Lake Superior in the Upper Peninsula of Michigan (see Fig. 2). Found in a semi-rural neighborhood of a scrub-riparian zone the bird was identified as a Tropical/Couch's Kingbird type (S. Haas, S. Hickman, pers. comm.). Subsequent to initial observations, many observers from all over Michigan - including members of the Michigan Bird Record Committee (MBRC) - traveled to see this bird. At one point, while being pursued by a Northern Shrike (*Lanius excubitor*) several observers heard the unknown *Tyrannus* individual (henceforth referred to as '*unkTyr*') make a series of twittery notes "tsit tsit tit tit" that had a slightly rolling quality similar to that described for *T. melancholicus*. (A. Byrne, J. Kaplan, pers. comm.)"

Fecal Sample Collection and DNA Extraction – On 2 November 2005 the

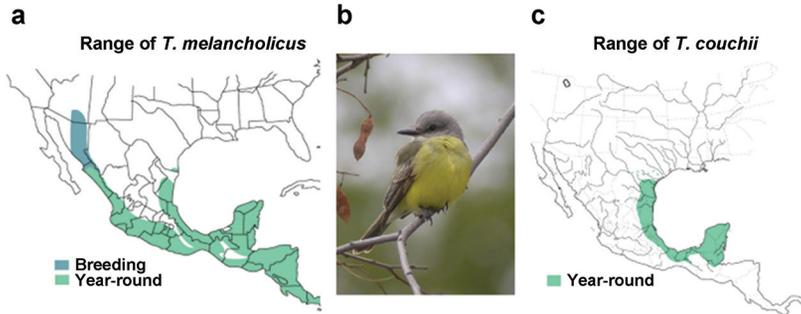


Fig. 1. a, Range map of Tropical Kingbird (*Tyrannus melancholicus*) distribution (from Stouffer *et al.* 1998); b, Photo of unknown kingbird (*Tyrannus* sp.) recorded near the mouth of the Au Train River, Alger County, Michigan USA (photo credit: S. Haas); c, Range map of Couch's Kingbird (*T. couchii*) distribution (from Brush 1999).

unkTyr was observed regularly “sallying” to and from the same perch to catch insects. One of the authors (S. Haas) spread a large plastic tarp beneath the perch, and soon the *unkTyr* deposited a single fecal sample (~100 mg) upon it. The fecal sample was placed in a plastic tube and stored at -20°C until DNA extraction. To avoid any possibility of contamination, DNA was extracted from the *unkTyr* fecal sample before reference *Tyrannus* samples arrived from other sources. DNA was extracted from ~25 mg of the *unkTyr* excrement using QIAamp® DNA Stool Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol for DNA isolation from human stool, with the exception of using half of an “InhibitEX” tablet during the extraction.

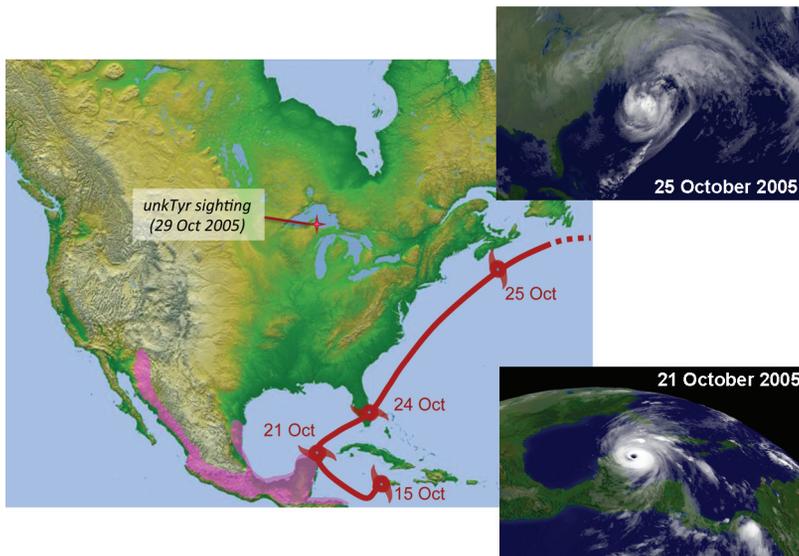


Fig. 2. Map of North America, showing the normal northern extent of the range of *T. melancholicus* (pink overlay on Central America), the track and timing of Hurricane Wilma (red line and whirls) and the location (crosshairs) and date when the unknown *Tyrannus* individual was recorded in Michigan's Upper Peninsula. Inset photos show satellite photographs of Hurricane Wilma on the dates indicated.

Table 1. Morphological and molecular data from reference specimens.

UMMZ#	<i>Tyrannus</i> sp.	Locale	Collection date	Morphological Measures ^a		Base positions	
				a/b	bill/wing	24	26
66501	<i>T. couchii</i>	Texas, USA	6-May-1930	0.769	13.3%	G	C
48860	<i>T. couchii</i>	Texas, USA	16-Apr-1909	0.833	14.3%	G	C
197476 ^d	<i>T. couchii</i>	Texas, USA	12-Apr-1933	0.750	12.8%	G	C
235346	<i>T. couchii</i>	Texas, USA	22-Apr-1998	NONE ^b		G	C
T-605	<i>T. melancholicus</i>	Venezuela	10-Feb-1989	NONE ^c		T	T
101091	<i>T. melancholicus</i>	Paraguay	4-Oct-1938	0.273	15.6%	T	T
108858	<i>T. melancholicus</i>	Paraguay	27-Jul-1940	0.273	14.4%	T	T
130638	<i>T. melancholicus</i>	Mexico	2-Jul-1950	0.727	16.4%	T	T
132797	<i>T. melancholicus</i>	Coast Rica	3-Sep-1950	0.462	14.3%	T	T
127684 ^d	<i>T. melancholicus</i>	Panama	6-Jan-1938	0.143	15.6%	T	T

a – See Results and Traylor (1979) for information on morphological measurements.

b – This specimen was a “shmoo” preparation, thus no bill data.

c – This DNA sample came from muscle tissue, specimen resides in Venezuela.

d – Sequences for these specimens are shown in the appendix.

Reference Samples – To establish reference DNA sequences from *T. melancholicus* and *T. couchii*, we obtained tissue samples from specimens in the University of Michigan Museum of Zoology bird collection. Tissues (9 toe pads, 1 muscle) were obtained from six *T. melancholicus* and four *T. couchii* individuals. Where available, species identity of specimens was morphologically confirmed with wing and bill measurements (Traylor 1979). The muscle sample had no voucher specimen available, and one specimen was a “shmoo” preparation so it lacked bill measurements. From the toe pad and muscle tissues of reference specimens we extracted DNA using DNEasy® Tissue Kit (Qiagen, Valencia CA, USA) protocol for total DNA isolation from animal tissues. See Table 1 for information on geographic origins, morphological measurements, and DNA sequence information of the museum specimens.

PCR Amplification and Sequencing – Using primer sequences previously reported to amplify mitochondrial and nuclear genes from avian genomes (Sorenson *et al.* 1999, Sorenson *et al.* 2003), we attempted to amplify six different fragments of DNA from the kingbird reference specimens and the DNA isolated from the fecal sample of the *unkTyr*. We used various PCR thermal profiles (including “touchdown” procedures) with the following mtDNA primer pairs (expected fragment lengths in parentheses): L537-H739 (202bp), L537-H774 (237bp), L537-H1251 (714bp), L1753-H1858 (103bp), and L4500-H5191 (691bp). See Sorenson *et al.* (1999) for primer sequences and mitochondrial map. We also tried to amplify a 618bp fragment from intron 9 of the nuclear gene coding for phosphoenolpyruvate carboxykinase (PEPCK) using primers and protocols from Sorenson *et al.* (2003).

Only primer pair L1753-H1858 successfully amplified fragments from the fecal sample and all of the tissue samples (including several that were >50 years old). This primer pair amplified a 103bp fragment of the mitochondrial gene encoding the 12S subunit of ribosomal RNA. Reactions were performed in 50 µL volumes with final concentrations of 0.2 mM dNTPs, 1.0 µM of forward

and reverse primers, 1 unit of Bullseye HS Taq polymerase (Midsci Inc.), Buffer II to the manufacturer's specifications, and 20.0 µg of BSA (bovine serum albumin: New England Biolabs). Amplified PCR products were resolved on 1.2% TBE agarose gels, excised with a scalpel and extracted from gel slices using Qiagen Gel Extraction Kit (Qiagen, Valencia CA, USA). Forward and reverse strands of excised PCR products were used for direct sequencing using Big Dye v.3.1 (Applied Biosystems, Foster City CA, USA). Sequencing reaction products were cleaned using Sephadex columns (Princeton Separations, Adelphia NJ, USA) and run on an ABI 3100-Avant genetic analyzer. Ambiguities (very few) in single-stranded sequences were reconciled between forward and reverse strands using the software program Geneious (Drummond *et al.* 2011). Primer sequences were trimmed from the sequences and each reconciled forward strand sequence was compared across all individuals from the known reference samples and the *unkTyr* sample.

RESULTS

Reference Specimens – Morphological measurements of five *T. melancholicus* and three *T. couchii* specimens confirmed their species identities according to morphological indices described by Traylor (1979). The indices used to compare the two species are derived from relative lengths of two primary feathers (primary 5 and primary 10) compared to a ratio of bill length to wing length. Traylor reported no raw values or averages for the 800 specimens he used, but the scatterplot of our specimens' morphological indices (Fig. 3) correctly separated the two species just as in Traylor (1979). The scatterplot shows the group representing *T. melancholicus* have bill/wing% between 14.5-17% and wing tip index less than 0.8, while the group representing *T. couchii* have bill/wing% between 12-16% and wing tip index greater than 0.7.

Sequence characteristics - Amplification products were recovered from all DNA extracts, including the DNA extracted from the fecal sample and those DNAs extracted from footpads of museum specimens (some more than 90 years old). All sequences were 103bp in length, and those from *T. melancholicus* samples matched exactly positions 408-510 of the 12S sequences published for *T. melancholicus* on Genbank (Accession # AF386462.1). Sequences from *T. couchii* differed from *T. melancholicus* at base positions 24 and 26 (see Table 1). There were no *T. couchii* sequences on Genbank for comparison. The *unkTyr* DNA sample matched perfectly the sequences of the *T. melancholicus* samples (thymines at base positions 24 and 26), differing from the *T. couchii* specimens (guanine and cytosine at base positions 24 and 26, respectively). Complete DNA sequences are shown in the Appendix (Genbank does not accession sequences of less than 103bp).

DISCUSSION

Using DNA isolated from avian feces collected non-invasively and DNA from reference specimens in museum collections, we identified a vagrant bird

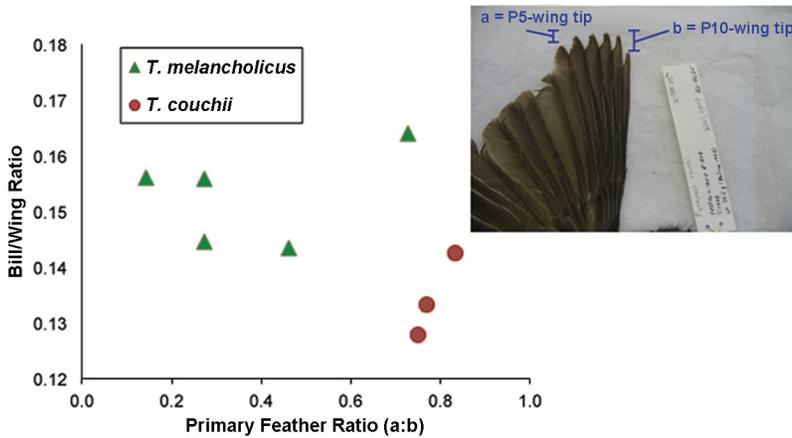


Fig. 3. Scatter plot showing the separation of *T. couchii* specimens from *T. melancholicus* specimens based on “wing tip index” (a/b in Table 1) vs. bill/wing length ratio, as originally described in Traylor (1979). Inset photo shows outer primaries of *T. couchii* and the measurements used to calculate the “wing tip index” (a/b in Table 1): the ratio of the difference between the wing tip and the 5th primary, divided by the difference between wing tip and the 10th primary.

to species and confirmed a first Michigan state record of *Tyrannus melancholicus*. To our knowledge, this is the first time any state record bird (Michigan or otherwise) has been established with DNA sequence data included as evidence. Feces have been used only sparingly for DNA isolation in past avian population studies, as PCR can often be inhibited due to exogenous and endogenous nucleases and inhibitory molecules remaining in the DNA extract (Regnaut *et al.* 2006). Interestingly, DNA from bird excrement with appreciable amounts of insect material has been reportedly difficult to amplify (Idaghdour *et al.* 2003). We were unable to retrieve any amplification of the DNA extract from feces without addition of BSA to the PCR cocktail – the only way we alleviated PCR amplification problems encountered from DNA of the feces of this insectivorous bird.

Our protocol, which combines a commercially available stool kit for DNA extraction and the addition of BSA for removal of PCR inhibitors (Al-Soud 2000), is one that should prove useful in many conditions where researchers have limited access to other sources for bird genetic material. For instance, in early 2008 a similar vagrant bird – putatively a White-crested Elaenia (*Elaenia albiceps*) – was observed on South Padre Island, Texas. This appearance inspired the birding community to a vigorous debate centered on whether to capture and examine the bird or to leave it uncaught and unknown. Our success with identifying this vagrant *T. melancholicus* provides an example of a non-invasive alternative for accurately documenting rare and difficult-to-identify birds.

Similar analyses of other vagrant individuals should similarly depend on comparisons with reference specimens for confirmation of species identities. In our analysis, we restricted our reference specimens to museum specimens that clearly fell into the morphological groupings delineated by Traylor (1979). To hinge species-identification on diagnostic genetic differences established from

a small sample could be considered problematic since the full range of genetic diversity in a species will remain unknown. However, two things increase our confidence in the species assignment performed in this study. First, our verification of morphological measurements from voucher specimens insures that the tissues were not acquired from misidentified individuals. Second, the reference specimens of *T. couchii* and *T. melancholicus* were collected over a long time span (90+ and 50+ years, respectively) and the *T. melancholicus* specimens were collected from a wide geographic range (from Paraguay to Mexico). Since each set of reference specimens was consistently identical within each species but was consistently different between species we have high confidence in the dependability of the observed diagnostic genetic differences between species.

What is the importance of documenting rare vagrant birds, especially if the vagrant species is not threatened in its native range? We recognize two important conservation reasons for using non-invasive methods to identify vagrant individuals. First, a changing global climate is predicted to significantly alter biologically-relevant weather phenomena (Easterling *et al.* 2000) – phenomena like the Atlantic hurricanes of 2005, the most significant storm season on record in the Atlantic Basin (Shein 2006). A possible explanation for the bird's appearance in Michigan is that the bird was transported northward by the powerful winds of Hurricane Wilma as it moved northward from the Yucatan Peninsula. Hurricane Wilma was the most intense hurricane ever recorded in the Atlantic basin (Shein 2006), and both its timing and path indicate it could have picked up this kingbird in the Yucatan peninsula around 18-21 October and continued to push it northward until the storm faded off the coast of Nova Scotia around 25-26 October (Fig. 2). It is probably not coincidence that the fall of 2005 was a remarkable season for vagrant birds in Michigan – seven new species were recorded in that four-month season, while all seasons from January 2000 to December of 2011 (*i.e.*, the 140-month period excluding fall 2005) brought only 17 other new species to Michigan (Fig. 4: MBRC online). Indeed north-to-south transfer of individuals like this kingbird won't likely generate significant changes in diversity or ecological integrity in either the donor or recipient habitats (Wehtje 2003), but similar east-west movements could have a greater likelihood of facilitating species invasions in the future (Patten and Marantz 1996).

The second reason conservation biologists should be concerned with identifying rare bird species through non-invasive methods like those used here is that rare birds generate a great deal of public awareness of birds and their conservation. In the case of this first state record of *T. melancholicus*, an estimated 30 observers from neighboring states traveled to Michigan's Upper Peninsula to view this bird. Likewise, a vagrant green-tailed towhee (*Pipilo chlorurus*) attracted nearly 200 visitors to a nearby area in the winter of 2006-2007 (L. Taccolini, pers. comm.). Amateur (and professional) birders can contribute significant resources to local economies, and definitively identifying rarities that might otherwise remain only tentatively identified can provide added value for the effort of these birders. These types of rare bird experiences hopefully hatch into heightened awareness of avian conservation issues, both local and global.

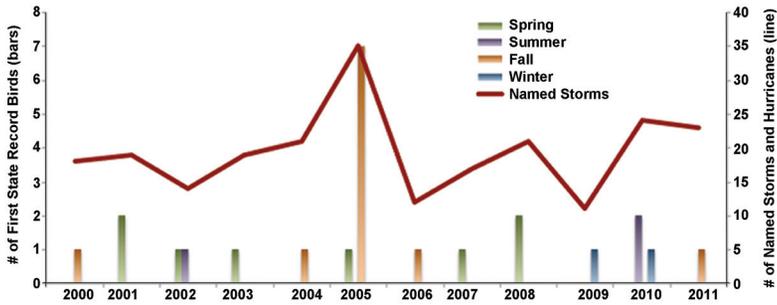


Fig. 4. Primary axis (bars) shows the numbers of Michigan first state record birds found in annual seasons from 2000-2011: Spring=Mar 1-May 30, Summer=Jun 1-July 30, Fall=Aug 1-Nov 30, Winter=Dec 1-Feb 28/29. Secondary axis (line) shows the total number of named storms (Tropical Storms + Hurricanes) recorded in the Atlantic Basin of each year. The unusual number of first state records in fall of 2005 occurs immediately after the most significant Atlantic Basin storm season on record.

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APPENDIX

Complete DNA sequences for the two *Tyrannus* species examined in this study. The two base pairs that differ between species are underlined in both (positions 24 and 26). DNA from the unknown *Tyrannus* sp. individual that appeared in Michigan's Upper Peninsula exactly matched the *T. melancholicus* sequence.

T. couchii – GCCTAGCCCTAAATCCTGATGTTGACCCTACCCAAACATC-
CGCCCGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTG-
GCGGTGCCCAAACCCAC

T. melancholicus – GCCTAGCCCTAAATCCTGATGTTTATCCTACCCAAA-
CATCCGCCCGAGAACTACGAGCACAAACGCTTAAAACTCTA-
AGGACTTGCGGTGCCCAAACCCAC