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DNA Barcoding of the First Recorded American Burying Beetle, *Nicrophorus americanus*, in Clark County, Arkansas

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Abstract

The American Burying Beetle (ABB), *Nicrophorus americanus*, is a red-listed endangered species under the Endangered Species Act (16 U.S.C. 1531 *et seq.)*. We serendipitously recorded 3 individuals of this species during a forensic study in the summer of 2013. These specimens represent the first known records for Clark County, AR and the southeastern-most record in the State since the extirpation of the species from the region in the late 1800's. Two males and one female were collected, photographed, sexed and measured. One male specimen was deceased upon discovery. The remaining two individuals were released. The U.S. Fish and Wildlife Service (USFWS) was notified of the accidental catch and death of an endangered species as required and the specimen was awarded to Ouachita Baptist University for further study. The deceased specimen was used for DNA barcode sequence analysis. A 400bp section of the cytochrome *c* oxidase I (COI) gene was amplified using gene specific primers and then sequenced using Sanger sequencing methods. Sequence analysis revealed the collected beetle to be 98.5% identical to the ABB voucher sequence and was 86% similar to other *Nicrophorus* species. Taken together the DNA sequence analysis results and taxonomic identification both support the identification of our specimen.

Introduction

We discovered three specimens of the American Burying Beetle (ABB), *Nicrophorus americanus* during a forensic study in the summer of 2013 in Clark County, Arkansas. Two males and one female were discovered on experimental pig and rat carrion over a three night period. Burying beetles are commonly associated with carrion (Scott 1998). A male specimen was found deceased on the second night and we realized it was an endangered ABB species. We immediately made contact with the United States Fish and Wildlife Service (USFWS) to report the accidental capture of an endangered species outside of its' known range. That specimen would be awarded to Ouachita Baptist University by letter from an Endangered Species specialist. Two subsequent individuals were noted in the next evening sample which were measured, sexed and released. Those captures were also reported. We suspended the field collections at that point to avoid the capture of further endangered ABB after consulting with the USFWS. Our specimens were captured in a mature growth forest just east of the City of Arkadelphia, AR. This location is consistent with the known preference of habitat as stated in Lomolino and Creighton (1996) and (Sikes 2005).

Reports of ABB capture are sporadically received by the USFWS but most tend to be inaccurate within the genus based on common field identification errors. Other closely related *Nicrophorus* species captured in our 2013 study included *N. orbicolis* and *N. tomentosus*. Our accidental capture report was met with initial skepticism until detailed descriptions and a crude phone camera photo were submitted (Figure 1). ABB are readily identifiable by their bright orange to red coloration on the head and pronotum, a feature lacking in the other *Nicrophorus* species (Sikes and Peck, 2000). ABB are also typically much larger than their closely related species and are the largest of the Silphid beetles in North America (Anderson and Peck 1985). The biology and distribution of the ABB is well established into four ecoregions within the United States, including the northwest region within the State of Arkansas (USFWS 2008).

A few previous studies have examined the genetic variation of the ABB in Arkansas (Kozol et al. 1994) and (Szalanski et al. 2000) and we knew that genetic comparisons were available for the ABB in the iPlant DNA Subway (Goff et al. 2011). We decided to confirm the identity of our specimen using DNA barcoding methods to eliminate any consideration of a false identification in the field. This was risky due to us

Figure 1. Deceased American Burying Beetle male on rat carrion in a bucket trap in Clark County, AR.

having only one individual to work with so we decided to run a test study with the closely related Nearctic burying beetle, *Nicrophorus orbicolis*. This study demonstrated that DNA extraction was best achieved using the material near the joints of the legs of the beetle. Our DNA barcoding effort was designed based upon the results of that study (Kelly and Jackson, unpub).

Materials and Methods

DNA extraction, PCR and Sequencing

DNA extraction and PCR were conducted using the DNA Barcode Amplification Kit (Carolina Biological Inc. Cat# 211385) without modifications. This kit was specially designed to be a robust DNA extraction procedure and was made under the recommendations of the International Barcode of Life project and the iPlant Collaborative Bar Code Project (Goff et al. 2011). A single beetle leg was used for DNA extraction. PCR was done using the Animal/Insect primer mix (Carolina Biological Cat# 211513). This primer mix is a cocktail of primers that target a 650 base pair segment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene. The insect specific primers LepF1A (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR1 (5'TAAACTTCTGGATGTCCAAAAAATCA-3') are included in this cocktail (Herbert et al. 2004). Both primers contained the M13 (5'-GTAAAACGACGGCCAGT-3') sequence on their 5' end. M13 specific primers were used for DNA sequencing. PCR amplicon purification and single direction sequencing were performed by GENEWIZ, inc. This sequence was submitted to NCBI GenBank (Accession ID: KX687862).

Sequence Analysis and Alignment

Sequence chromatograms were edited and assembled using the iPlant: DNA subway editing tool (http://dnasubway.iplantcollaborative.org/, Goff et al. 2011). These alignments were examined by eye to detect potential base calling errors, particularly at the beginning and ends of traces. Potential errors were checked in the trace files and corrected (shortened) as necessary. Figure 1 provides the shortened trace file submitted for comparison. Sequence alignments and similarity were generated using MUSCLE [v. 3.8.31], (Robert, 2004) as implemented by DNA Subway. Additional, alignments, comparisons and dot plot were generated directly using the Basic Local Alignment Search Tool [BLAST 2.3.1] (Altschul et al. 1997) on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Our BLAST results were compared to the *Nicrophorus americanus* voucher specimen.

Results and Discussion

DNA Barcode Analysis

DNA barcoding uses a 650 base pair region of DNA associated with the mitochondrial cytochrome oxidase subunit 1 gene (COI) to create a genetic DNA barcode (Ratnasingham and Herbert 2013, Sikes and Venables 2013). Identification is achieved by comparing the sequenced data or genetic barcode to a reference library containing known taxa or voucher sequences (Ratnasingham and Herbert 2007).

A 600 base pair region of the Clark County ABB specimen was isolated and sequenced using conventional DNA barcode protocols (Goff et al. 2011). However this sequence was trimmed to a 352 base pair section (Figure 2) which was used for analysis and identification. Trimming was performed due to several misreads at the ends of each tail of the ABB sequence. While there was reliable data with good homology in these areas the occasional presence of misread basses, designated with an n, would potentially make this data un-reliable. We did not feel confident in including misreads in our submission.

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>TTATTTTATTTTTGGTGCCTGAGCAGGAATAGCCGGGATATCACTTAGAATATTAATCCGAGTAGAGTTA
AGAACCCCAGGAACTTTACTTGGTGATGACCAAATGTATAATGTTATTGTAACTGCTCATGCATTTATCA
TAATTTTTTTTATAGTAATACCTATTGTAATTGGTGGATTTGGAAATTGACTAGTACCCCTTATACTAGG
AGCCCCTGATATAGCTTTCCCTCGATTAAATAATATAAGATTTTGATTATTACCCCCATCATTATCATTG
TTATTAATCTCTAGAATAGTAGAAAGAGGAGCTGGCACAGGTTGAACAGTGTACCCCCCACTCTCAGCCA
ATATTGCTCATAGAGGATCTTCTGTAGATTTAGCAATTTTTAGATTACATTTAGCTGGTATTTCGTCAAT
TCTTGGAGCAGTAAATTTTATTACAACAGTAATTAATATACGATCACCAGGGATAACCTTTGATCGAATA
CCATTATTTGTATGATCAGTTGCTATTACTGCTTTATTACTCCTTTTATCTTTACCTGTACTAGCAGGAG
CTATTACTATATTACTTACAGATCGAAATTTAAATACATCCTTTTTTGATCCCGC
```

Figure 2. FASTA trimmed data for the Clark County 2013 American Burying Beetle specimen (GenBank ID: KX687862).

However, it should be noted that misreads are common at the beginning and ends of DNA sequenced with Sanger sequencing methods. Due to the scarcity of our material, we were unable to submit additional samples for sequencing.

BLAST analysis revealed this sequence (GenBank ID: KX687862) to be 98.6% identical to the American Burying Beetle voucher sequence (GenBank ID: EU147412) (Figure 3).

It should be noted that the gene bank voucher sequence was submitted as the entire COI gene and is over 2,000 base pairs long, however only the first 400 base pairs of this gene are commonly used for DNA barcode analyses (Ratnasingham and Herbert 2013, Sikes and Venables 2013). Interestingly, other submitted *Nicrophorus* species COI voucher sequences have been trimmed to only include the COI gene. This may be due to the endangered status of the ABB. No other *Nicrophorus* species has a sequence similarity above 87% when compared to our sequence (Figure 4).

Conclusions

The aim of this study was to confirm that we had discovered and properly identified three specimens of the endangered American Burying beetle, *Nicrophorus americanus* in Clark County, AR in the summer of 2013. Based upon the strong physical characteristics used during the 2013 field and lab identification of the Clark County specimens and the strong COI DNA sequence correlation between our specimen and the gene bank voucher specimen, we conclude that the three individuals captured in Clark County in 2013 were the endangered American Burying Beetle, *Nicrophorus americanus*. DNA barcoding is an effective tool which assists in species identification and reduces potential errors related to taxonomic impediment. The USFWS has indicated that further regional sampling will take place in 2015 to detect any persisting Clark County ABB populations. Further studies by Ouachita Baptist University biologists have

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Query  1    GATATAGCTTTCCCTCGATTAAATAACATAAGATTTTGATTATTACCCCCATCATTATCA  60
           ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  218  GATATAGCTTTCCCTCGATTAAATAATATAAGATTTTGATTATTACCCCCATCATTATCA  277

Query  61   TTGTTATTAATCTCTAGAATAGTAGAAAGAGGAGCTGGCACAGGTTGAACAGTGTACCCC  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278  TTGTTATTAATCTCTAGAATAGTAGAAAGAGGAGCTGGCACAGGTTGAACAGTGTACCCC  337

Query  121  CCACTATCAGCCAATATTGCTCATAGAGGATCTTCTGTAGATTTAGCAATTTTTAGATTA  180
           ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  338  CCACTCTCAGCCAATATTGCTCATAGAGGATCTTCTGTAGATTTAGCAATTTTTAGATTA  397

Query  181  CATTTAGCTGGTATTTCATCAATTCTTGGAGCAGTAAATTTTATTACAACAGTAATTAAT  240
           |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  CATTTAGCTGGTATTTCGTCAATTCTTGGAGCAGTAAATTTTATTACAACAGTAATTAAT  457

Query  241  ATACGATCACCAGGGATAACCTTTGATCGAATACCATTATTTGTGTGATCAGTTGCTATT  300
           |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  458  ATACGATCACCAGGGATAACCTTTGATCGAATACCATTATTTGTATGATCAGTTGCTATT  517

Query  301  ACTGCTTTACTACTCCTTTTATCTTTACCTGTACTAGCAGGAGCTATTACTATATTACTT  360
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  ACTGCTTTATTACTCCTTTTATCTTTACCTGTACTAGCAGGAGCTATTACTATATTACTT  577

Query  361  ACAGATCGAAATTTAAATACATCTTTTTTTGATCC  395
           |||||||||||||||||||||||| ||||||||||
Sbjct  578  ACAGATCGAAATTTAAATACATCCTTTTTTGATCC  612
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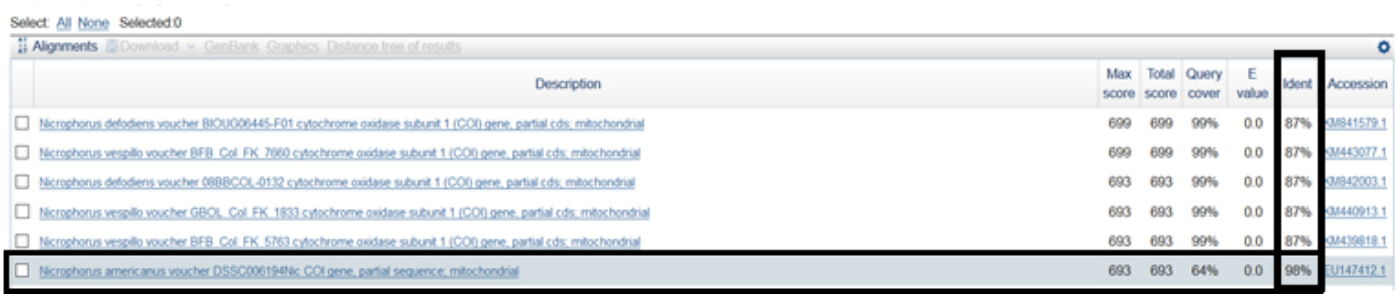
Figure 3. Sequence alignment with the Clark County ABB specimen and the gene bank ABB voucher specimen (EU147412). A 352 base pair sequence alignment of the Clark County and voucher ABB sequences is shown. Alignments were performed using BLAST. Analysis shows a 98.6% similarity between the two specimen sequences. Differences in sequence are noted in bold.



Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Nicrophorus defodiens voucher BIOUG06445-F01 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	699	699	99%	0.0	87%	GM941579.1
☐	Nicrophorus vespilloides voucher BFB_Col_FK_7660 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	699	699	99%	0.0	87%	GM443077.1
☐	Nicrophorus defodiens voucher 08BBCOL-0132 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	693	693	99%	0.0	87%	GM942003.1
☐	Nicrophorus vespilloides voucher GBOL_Col_FK_1833 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	693	693	99%	0.0	87%	GM440913.1
☐	Nicrophorus vespilloides voucher BFB_Col_FK_5763 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	693	693	99%	0.0	87%	GM439818.1
☐	Nicrophorus americanus voucher DSSC006194Nic COI gene, partial sequence; mitochondrial	693	693	64%	0.0	98%	EU147412.1

Figure 4. BLAST result using the Clark County ABB sequence as the query. Analysis reveals a 98% similarity with the ABB voucher sequence (highlighted). Further, the Clark County ABB sample reveals an 87% sequence similarity with other *Nicrophorus* species, supporting the membership of the Clark County specimen within the genus.

been postponed pending a response to an outstanding 2014 endangered species permit request.

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