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Erin M. Borland
Colorado State University - Fort Collins, erin.borland@colostate.edu

Daniel A. Hartman
Colorado State University - Fort Collins, daniel.anthony.hartman@colostate.edu

Matthew W. Hopken
USDA-APHIS NWRC, mhopken@rams.colostate.edu

Antoinette J. Piaggio
USDA APHIS Wildlife Services, Toni.J.Piaggio@aphis.usda.gov

Rebekah C. Kading
Colorado State University - Fort Collins, rebekah.kading@colostate.edu

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Short Communication

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Technical Limitations Associated With Molecular Barcoding of Arthropod Bloodmeals Taken From North American Deer Species

Erin M. Borland[1,3], Daniel A. Hartman[1], Matthew W. Hopken[1,2], Antoinette J. Piaggio[2], and Rebekah C. Kading[1]

[1]Colorado State University, 200 W Lake Street, Fort Collins, CO 80523-1690, [2]USDA/APHIS/WS National Wildlife Research Center, 4101 LaPorte Ave., Fort Collins, CO 80521, and [3]Corresponding author, e-mail: erin.borland@colostate.edu.

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Abstract

Accurate species-level identification of the source of arthropod bloodmeals is important for deciphering blood feeding patterns of field-collected specimens. *Cytochrome c oxidase I* (*COI*) mitochondrial gene sequencing has been used for this purpose; however, species resolution can be difficult to obtain from certain vertebrate genera, including *Odocoileus*. Sanger sequencing of mitochondrial genes was employed to identify the bloodmeal source of wild-caught mosquitoes trapped in Greeley, Colorado. Initial sequencing of the *COI* gene of mitochondrial DNA in bloodmeals was inadequate for species-level resolution of bloodmeals from deer in the genus *Odocoileus*, with current databases returning low fidelity matches to multiple genera. The use of the hypervariable D loop of the control region provided species-level identification of white-tailed deer (Order: Artiodactyla, Family: Cervidae, *Odocoileus virginianus*); however, taxonomic identification was successful only to genus for mule (*O. hemionus hemionus*) and black-tailed deer (*O. hemionus columbianus*). We advocate the use of multiple loci for bloodmeal analysis and the buildout of available databases to include multiple mitochondrial reference genes for reliable host species identification.

Key words: bloodmeal identification, *Odocoileus*, deer, Arbovirus ecology, mosquito

Approximately a quarter of pathogen outbreaks in the 20th century were spread by hematophagous arthropod vectors, showcasing the important role of vector surveillance and ecology to public health practice (Jones et al. 2008). Deer can act as amplification hosts for a number of emerging or invasive viruses, including arboviruses. For example, epizootic hemorrhagic disease virus (EHDV) is a significant arboviral pathogen of white-tailed deer and cattle, transmitted by biting midges *Culicoides* spp. Latreille 1809 (Diptera: Ceratopogonidae). Multiple serotypes of EHDV currently circulate in the United States, presenting a health threat to domestic and wild ruminants (Shope et al. 1960, Ruder et al. 2016). Current data indicate that white-tailed deer *Odocoileus virginianus* Zimmermann 1780 (Artiodactyla: Cervidae) are also highly susceptible to Rift Valley fever virus (RVFV) infection and could serve as an amplification host if this virus is introduced to the United States (Kakani et al. 2010; Hartley et al. 2011; Golnar et al. 2014, 2018; Wilson et al. 2018). Modeling further suggests that deer could enhance spillover of some pathogens into human populations because of their close proximity to high-density urban areas (Kakani et al. 2010); deer tend to interface with humans frequently in these areas and could potentially be exposed to arthropods with promiscuous feeding habits, promoting the spread of arthropod-borne disease. Evidence also supports the susceptibility and exposure of deer to a number of additional arboviruses around the globe (Hubalek et al. 2014). Thus, reliable techniques for uncovering the deer/arthropod vector network is of critical importance from a One Health perspective (Sinclair 2019). If we understand more about these transmission dynamics, we can better protect human populations from spillover of disease.

Molecular identification of vector bloodmeal source to the species level has become an increasingly important tool to understand the transmission dynamics of arthropod-vectored pathogens (Kent 2009). Nucleotide-based approaches are widely used and a multitude of published PCR assays targeting mitochondrial DNA (mtDNA) are available (Kent 2009, Brinkmann et al. 2016, Logue et al. 2016). Concerns about the use of mtDNA sequences for species delimitation have been raised because ancestral polymorphisms can persist long after species divergence due to incomplete lineage sorting, as well as introgression between species dating back millions of years (Mallet and Willmott 2003). North American deer of the family *Cervidae* includes the genus *Odocoileus*, with

two species *O. virginianus* (white-tailed deer) and *Odocoileus hemionus* Rafinesque 1817 (Artiodactyla: Cervidae) (mule deer), each with multiple subspecies (Wilson and Reeder 2005). Genetic studies including both species have identified instances of limited divergence and shared haplotypes which has been interpreted in two ways: historic introgression (Carr et al. 1986), and incomplete lineage sorting (Cronin et al. 1988). Reports of introgression between these two species has found *O. virginianus* mtDNA in *O. hemionus* individuals (Carr et al. 1986), and later studies suggested that interspecies gene flow is not unidirectional (Ballinger et al. 1992). The complexity of shared genetic history and potential for recent hybrids can make it challenging to separate the species with small fragments of mtDNA as used for barcoding of bloodmeals from arthropods.

As part of a larger study on mosquito community composition and blood feeding behavior, we identified blood meals from a diversity of mosquito species and locations in northern Colorado (Hartman et al. 2019). Using existing and widely-used protocols, we identified some blood meals to the family *Cervidae*, but were unable to obtain greater resolution to genus or species. Here, the utility of two mtDNA loci, COI and the d-loop control region (CR), to detect the bloodmeal source of mosquitoes that fed on deer was examined. We then discuss the challenges and limitations associated with these loci for identification of mosquito bloodmeal host sources.

Materials and Methods

Engorged mosquitoes were collected by CDC light traps in 2016 in Weld County, Colorado, USA (Hartman et al. 2019). Mosquitoes were morphologically identified using available taxonomic keys (Darsie and Ward 2005). DNA was extracted from engorged mosquito abdomens using the Qiagen DNA Investigator kit (Qiagen, Valencia, CA) as described previously (Hartman et al. 2019).

DNA samples extracted from muscle tissue of morphologically and genetically identified deer collected in Oregon between 2008 and 2010 were received from the USDA/APHIS/WS National Wildlife Research Center (Fort Collins, CO) (Table 2) (Hopken et al. 2015).

PCR amplification was conducted using GoTaq Green Hot Start Master Mix (Promega, Madison, Wisconsin, USA) in 25 µL reaction volumes (Table 1). The COI primer cocktails were prepared using a 1:1:2 ratio as described previously (Ivanova et al. 2006). The primer H16501 paired most closely with available reference sequences and was utilized (Table 1) (Purdue et al. 2000). Amplification products were verified by electrophoresis on 1.2% agarose gels (Invitrogen, Carlsbad, CA), and were purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands).

Sanger sequencing was performed by Quintara Biosciences (Berkeley, CA). *COI* sequencing was performed using primers targeting M13-tail motifs present on the amplification primers (Table 1). Raw sequences were trimmed and analyzed for call quality and nucleotide polymorphisms using Geneious 11.1 software (Biomatters Ltd., Auckland, New Zealand). Sequences were aligned by Pairwise/Multiple alignment using the Geneious Alignment algorithm, global alignment with free end gaps, and a 93% similarity cost matrix. Sequences greater than 200 nucleotides in length were submitted to National Center for Biotechnology Information (NCBI) GenBank (Table 2).

CR sequences were generated for a single control sample (WG091117-016; *Odocoileus hemionus hemionus*) and compared with the corresponding reference sequence from GenBank (Accession KP308259.1). Identity was confirmed, and previously published CR sequences for the controls were downloaded from GenBank for analysis (Table 2) (Hopken et al. 2015).

Consensus sequences were used to query the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007), and/or the nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al. 1990). BOLD 'Species-level Barcode Records' were queried in order to return the best match to the species level in the curated database; all sequences returned a match using this algorithm. BLASTn searches were optimized for highly similar sequences (megablast). Sequence identity by species (98–100% identity), genus (91–97% identity), and family (88–90% identity) was determined as described previously (Hebert et al. 2003, Kent 2009).

Results

Archived DNA from engorged field-collected mosquitoes (Hartman et al. 2019) matched *COI* sequences from ungulates in the family

Table 1. Primer target, sequence, and cycling parameters for amplification of COI and CR mtDNA amplicons

Target region	Primer source	Size (nt)	Primer	Sequence	Cycling parameters
Cytochrome c oxidase I	Ivanova et al. (2007)	658	VF1_t1	TGT AAA ACG ACG GCC AGT TCT CAA CCA ACC ACA AAG ACA TTG G	95°C for 5 min; 5 cycles of 95°C for 30 s, 52°C for 40 s, 72°C for 60 s; 35 cycles of 95°C for 30 s, 54°C for 40 s, 72°C for 60 s; 72°C for 10 min; hold at 4°C.
			VF1d_t1	TGT AAA ACG ACG GCC AGT TCT CAA CCA ACC ACA ARG AYA TYG G	
			VFLi_t1	TGT AAA ACG ACG GCC AGT TCT CAA CCA ACC AIA AIG AIA TIG G	
			VR1d_t1	CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GGC CRA ARA AYC A	
			VR1_t1	CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GGC CAA AGA ATC A	
			VRli_t1	CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GIC CIA AIA AIC A	
			M13F	TGT AAA ACG ACG GCC AGT	Sequencing primer
			M13R	CAG GAA ACA GCT ATG AC	Sequencing primer
mtDNA control region	Purdue et al. (2000)	685	H16501A	ATG GCC CTG TAG AAA GAA C	95°C for 5 min; 35 cycles of 93°C for 30 s, 52°C for 40 s, 72°C for 60 s + 3 s/cycle; 72°C for 5 min; hold at 4°C.
			L15926	TAC ACT GGT CTT GTA AAC C	

Table 2. PCR and sequencing results for deer samples and controls for COI and CR genes

Sample/control ID	Mosquito species	Final species identification	COI PCR				CR PCR		
			Top BOLD result	% Match	Top BLAST result	% Match	GenBank Accession #	Top BLAST result	% Match
397	Aedes species (Meigen, 1818)	*Odocoileus virginianus*	No match	N/A	*Odocoileus virginianus* (predicted)	99.1	MN117131	*Odocoileus virginianus*	100.0
479	*Aedes dorsalis* (Meigen 1830)	*Odocoileus virginianus*	*Elaphurus davidianus*	89.7	*Odocoileus virginianus* (predicted)	98.4	MN117132	*Odocoileus virginianus*	99.8
606	*Aedes vexans* (Meigen 1830)	*Odocoileus hemionus*	*Elaphurus davidianus*	89.5	*Odocoileus virginianus* (predicted)	100.0	MN117133	*Odocoileus hemionus*	100.0
628	*Aedes vexans*	*Odocoileus hemionus*	*Elaphurus davidianus*	89.6	*Odocoileus virginianus* (predicted)	98.9	MN117134	*Odocoileus hemionus*	100.0
3343	*Aedes dorsalis*	*Odocoileus virginianus*	*Elaphurus davidianus*	89.7	*Odocoileus virginianus* (predicted)	98.4	MN117135	*Odocoileus virginianus*	99.8
3345a	*Aedes vexans*	*Odocoileus virginianus*	*Elaphurus davidianus*	89.7	*Odocoileus virginianus* (predicted)	99.0	MN117136	*Odocoileus virginianus*	100.0
3392a	*Aedes vexans*	*Odocoileus virginianus*	*Elaphurus davidianus*	89.5	*Odocoileus virginianus* (predicted)	99.6	MN117137	*Odocoileus virginianus*	100.0
3392d	*Aedes vexans*	*Odocoileus virginianus*	*Capra aegagrus hircus* (Linnaeus, 1758)	89.3	*Odocoileus virginianus* (predicted)	98.3	MN117138	*Odocoileus virginianus*	100.0
WG080410-001	N/A	*Odocoileus virginianus ochrourus*	Poor sequence quality		Poor sequence quality		KP308270.1	*Odocoileus virginianus*	98–100
WG100428-010	N/A	*Odocoileus virginianus ochrourus*	Poor sequence quality		Poor sequence quality		KP308271.1	*Odocoileus virginianus*	98–100
WG080827-001	N/A	*Odocoileus virginianus leucurus*	No band by PCR		No band by PCR		KP308266.1	*Odocoileus virginianus*	98–100
WG080520-001	N/A	*Odocoileus virginianus leucurus*	Poor sequence quality		Poor sequence quality		KP308262.1	*Odocoileus virginianus*	98–100
WG081103-005	N/A	*Odocoileus hemionus columbianus*	*Odocoileus virginianus*/ *Odocoileus hemionus*	98.7	*Odocoileus virginianus*/ *Odocoileus hemionus*	97–98	KP308268.1	*Odocoileus virginianus*/ *Odocoileus hemionus*	98–100
WG081103-021	N/A	*Odocoileus hemionus columbianus*	Poor sequence quality		Poor sequence quality		KP308265.1	*Odocoileus hemionus*	98–100
WG091117-013	N/A	*Odocoileus hemionus hemionus*	*Odocoileus virginianus*/ *Odocoileus hemionus*	100.0	*Odocoileus virginianus*/ *Odocoileus hemionus*	99–100	KP308247.1	*Odocoileus virginianus*/ *Odocoileus hemionus*	98–100
WG091117-016	N/A	*Odocoileus hemionus hemionus*	*Odocoileus virginianus*/ *Odocoileus hemionus*	99.9	*Odocoileus virginianus*/ *Odocoileus hemionus*	99.0	KP308259.1	*Odocoileus hemionus*	98–100

Cervidae; however, the blood hosts that these mosquitoes fed upon were not readily identifiable beyond family (Table 2) (Ivanova et al. 2007). Six sequences returned low identity matches (~89%) using the BOLD database to species not present in the area (*Elaphurus davidianus* Milne-Edwards 1872 [Artiodactyla: Cervidae] Père David's deer). The BLAST database returned a high-quality match to a single, predicted white-tailed deer sequence for all six samples (GenBank XR 002310491.1). The percentage identity to white-tailed deer was promising since the Northern Rocky Mountain white-tailed deer (*O. virginianus dacotensis*) is known to inhabit the area, but we questioned the accuracy of this result because mule deer were commonly observed at the collection sites (Colorado Parks and Wildlife, Department of Natural Resources).

Given our need to identify the field samples to the correct species, extracted DNA controls belonging to North American deer were obtained and assays were validated for their ability to correctly identify these controls (Table 2). Control DNA was amplified using the *COI* primer set with limited success: sequence greater than 200 nucleotides in length and with clean chromatograms free of distortion or ambiguous bases was only obtained for three samples despite multiple attempts. The BLAST and BOLD databases returned matches to both white-tailed and mule deer at 98–100% identity for these three samples. This would be considered robust species identification (Hebert et al. 2003, Kent 2009), although resolution of species status could not be achieved at this locus. In contrast, white-tailed deer control CR sequences produced 100% matches to other white-tailed deer, with the closest secondary matches to mule deer at 96–97%, allowing for adequate species delimitation (Table 2). Black-tailed deer (*O. hemionus columbianus*) controls matched to a number of sequences of this species in the database, but also produced two matches to white-tailed deer sequence at 98%–99%, likely due to mitochondrial introgression from black-tailed deer (GenBank #s KP308229.1, KP308236.1) (Hopken et al. 2015). Mule deer controls returned a mixed list of white-tailed deer, mule or black-tailed deer with 98–100% identity. The CR assay was able to clearly identify white-tailed deer with adequate species delimitation, but identification of the mule and black-tailed deer subspecies was restricted to the genus level.

When field samples were tested with CR primers, six samples had 99–100% identity to white-tailed deer with secondary matches to mule deer at 96–97%. Based on the higher level of sequence homology and adequate separation of species, we identified these as white-tailed deer. The remaining two samples matched to mule deer with 100% identity, but also returned a mixed list of both mule and white-tailed deer at 99% identity. This result closely resembles those generated for the mule deer controls, indicating that the identification of these samples as mule deer is likely given the fact that these samples were not white-tailed deer and only these two species are present in the area.

To ensure that Colorado deer species were not absent or critically underrepresented in the BOLD and GenBank databases, a query was performed (2 March 2020) (Supp Table 1 [online only]). Adequate representation for COI was found in both databases, although the vast majority of animals in the BOLD database originated in Canada. A very large number of CR sequences were found in the GenBank database, indicating that this locus is frequently used for the identification of deer species.

Discussion

We report here ambiguities associated with arthropod bloodmeal identification and obtaining species identification of deer from field-collected samples. In our analysis of the mosquito bloodmeals from the family *Cervidae* in Colorado, molecular identification was complicated by mtDNA genetic introgression and lack of monophyly and insufficient resolution provided by COI molecular markers. Although our study focused on COI, any conserved region of the mtDNA, such as cytochrome b, would also be faced with the same challenges associated with mitochondrial introgression between species, leaving only hypervariable regions capable of molecular delimitation (Hopken et al. 2015). The inability to resolve *Odocoileus* sequences using BOLD remains unclear because there appears to be adequate representation of the target gene region from these taxa. Identification was ultimately attained with primers targeting the hyper-variable region of the CR. The utility of this locus was further supported by the plentitude of CR sequences available in the GenBank database for both species. The CR assay was able to identify white-tailed deer with 3–4% interspecific divergence from mule and black-tailed deer. Black-tailed deer controls returned self-matches with the exception of a single white-tailed deer sequence. Mule deer were identified less successfully, with controls returning a mixed list of white-tailed deer and mule deer with 98–100% identity. In some geographical regions, these species are not sympatric and hence identity could be achieved. Despite these challenges, the CR clearly had more success in identification of western North American deer species than COI sequencing.

The difficulties with *Odocoileus* identifications exemplify larger issues with single-gene pan-species barcoding approaches for bloodmeal identification (Hebert et al. 2004). Early critics of the single-gene pan-species approach identified limitations of using mtDNA to infer species boundaries, including incomplete lineage sorting, sex-biased gene flow, selection on mtDNA, introgression, and paralogy resulting from transfer of mtDNA gene copies to the nucleus (Bensasson et al. 2001, Ballard and Whitlock 2004, Moritz and Cicero 2004). Multiple studies have established the dual directional flow of mtDNA between white-tailed deer and black-tailed or mule deer, and hybrids of the two species are known to be fertile (Carr et al. 1986, Derr et al. 1991, Ballinger et al. 1992, Cathey et al. 1998). Despite these challenges, recent studies utilizing the CR for intraspecific identification of white-tailed, black-tailed, and mule deer illustrate the potential usefulness of this region for interspecies delimitation (Latch et al. 2009, Hopken et al. 2015). Here, we have shown some utility of this locus, albeit with limitations.

Criticisms of barcoding have arisen due to complications associated with other vertebrate groups (Moritz and Cicero 2004) and have identified numerous pitfalls associated with DNA barcoding. These include multiple instances in which the intra- and interspecific differentiation among North American bird species was not always concordant with molecular (COI) barcoding criteria. For some taxa, deposition of 'COI-like' pseudogene sequences on GenBank has posed a problem for accurate species identification, as these sequences likely represent nuclear copies of mitochondrial derived genes (numts) and contain insertions, deletions, and stop codons (Buhay 2009). A 'one size fits all' approach to molecular barcoding of all species is unlikely to be feasible due to the incredible genetic and biological diversity present across ecosystems (Moritz and Cicero 2004). Identification of 'blind spots' in commonly used PCR assays is vital to progress in the field of DNA barcoding. We identified one such gap and suggested an alternate assay to COI that might be used for the delimitation of deer species.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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Supplementary Table 1: Available Sequences in BOLD and GenBank Databases

	Odocoileus virginianus	*Odocoileus hemionus*
Complete Genome- GenBank (BLAST) Records:	1 (NC_015247.1)	1 (NC_020729.1)
Complete Mitochondrion- GenBank (BLAST) Records:	14	3
COI- BOLD Records:	34*	14**
COI: GenBank (BLAST) Records:	20	8
d-loop CR: GenBank (BLAST) Records:	352***	624****

*19 BOLD-specific records from Canada, remainder mined from GenBank

18 records from Canada, 1 record from Mexico, 15 records from unspecified location

** 10 BOLD-specific records from Canada, remainder mined from GenBank or ATCC

10 records from Canada, 3 records from unspecified location, 1 from tissue culture

*** An additional 26 sequences predicted by automated computational analysis are available

**** D-loop sequences from across the American West, including CO