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Short Communication

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Genetic Diversity of *Culicoides stellifer* (Diptera: Ceratopogonidae) in the Southeastern United States Compared With Sequences From Ontario, Canada

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Abstract

Much of the bluetongue (BT) and epizootic hemorrhagic disease (EHD) research in North America focuses on white-tail deer and *Culicoides sonorensis* (Wirth & Jones) (Diptera: Ceratopogonidae), though several other biting midge species have been suggested as vectors. *Culicoides stellifer* (Coquillett) has been associated with hosts susceptible to hemorrhagic disease (HD), and more recently, specimens from Florida have tested positive for EHD and BT viral RNA. If *C. stellifer* is acting as a vector, this could have an impact on the distribution of HD in North America. To determine if gene flow is occurring across the range of *C. stellifer* within the southeast United States, a mitochondrial haplotype analysis was performed using the *COI* gene. Our haplotype network showed no population structure in *C. stellifer* from Florida, Texas, and South Carolina, as the overall genetic divergence between these sites was equal to the genetic divergence within each. We also compared these haplotypes to published sequences of *C. stellifer* collected in Ontario, Canada. Surprisingly, the genetic diversity of the flies from Ontario was two times greater than what was observed between the southeast U.S. collection sites. This considerable divergence could be evidence of a cryptic species. A better understanding of the connectivity between *C. stellifer* populations across all of North America will give insight into the distribution of HD. Our results show that gene flow is occurring between sites in the southeastern United States and potentially throughout the eastern distribution of the species.

Key words: hemorrhagic disease, biting midge, vector, haplotype network, population genetics

The population dynamics of insect vectors can be used to determine potential routes of pathogen transmission (Kozakiewicz et al. 2018). Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) can cause severe symptoms and death in deer and sheep (Gibbs and Greiner 1994) and subclinical or asymptomatic infections in goats and cattle (Spickler and Roth 2006). The estimated annual economic loss due to these viruses is 3 billion USD worldwide (Rushton and Lyons 2015) and 125 million USD in the United States (Tabachnick 1996). These viruses are transmitted by minute biting midges in the genus *Culicoides* Latreille. Midges show wind-mediated dispersal patterns, and without geographical barriers, maintain connectivity over large geographical distances (Onyango et al. 2015b, Jacquet et al. 2016b). Often, the population dynamics of the vector can be used to better understand the factors linked to transmission (McCoy 2008). To date, several studies on the genetic structure of *Culicoides* spp. have found high levels of gene flow with no spatial or temporal structuring in Europe, Africa, and Australia (Pili et al. 2010; Onyango et al. 2015a, 2016; Jacquet et al. 2016a), but such studies have not been conducted in the United States. Even if there is not a direct correlation between midge movement and emergence of the virus, the population genetic structure of a vector species can help to decipher other potential factors leading to disease outbreaks (Jacquet et al. 2015, Onyango et al. 2016). The analysis of population genetics of insect vectors can also prove very helpful to the implementation of future control strategies.

While *Culicoides sonorensis* (Wirth & Jones) is the only confirmed vector of EHDV and one of only two confirmed BTV vectors in the United States, this species is rare in some areas of the eastern United States where disease persists (Grogan and Lysyk 2015). *Culicoides stellifer* (Coquillett) is one of the most common biting midge species throughout the Nearctic—ranging from Ontario to Nova Scotia in Canada and through the entire continental United

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States, except Washington and Oregon (Blanton and Wirth 1979, Borkent and Grogan 2009). If vector competency extends throughout this species' geographic range, many areas of the United States and Canada could be at risk of outbreaks. There has been a steady range expansion of hemorrhagic disease (HD) into '*C. sonorensis*-free areas' (Stallknecht and Poulson 2019), and *C. stellifer* could be enabling this expansion either through the dispersal of infected midges or when infected hosts are moved to areas where this species is already established.

In the United States, relatively few studies have been conducted focusing on the vector capacity of *C. stellifer*, and each of these studies was conducted using a population from an isolated geographic area (in comparison to the overall distribution of this species). Wieser-Schimpf et al. (1993) did not detect BT viral RNA from 125 pools of *C. stellifer* from a population in Louisiana; however, EHDV and BTV have been detected in field-collected individuals in Florida (McGregor et al. 2019). *Culicoides stellifer* from Alabama have been shown to harbor live BTV when experimentally infected via thoracic injection, though viral survival rates were low (Mullen et al. 1985). This species has also been demonstrated to feed on vertebrate hosts that are susceptible to HD (Blanton and Wirth 1979, McGregor et al. 2018). Studies on other vector groups have found that with increasing genetic distance, traits such as vector competence and host association become increasingly variable (McCoy et al. 2001, Beebe et al. 2005). By investigating the overall genetic similarity of *C. stellifer* from populations used in these earlier works, we can assess the probability that these individuals share such traits. If the method for dispersal and migration of *C. stellifer* are similar to what has been observed in other *Culicoides* species, we would expect a high level of connectivity and gene flow between purported populations.

Materials and Methods

Midge Collecting

Adult *Culicoides* were collected using a UV CDC miniature light trap (Bioquip 2836BQ) suspended near livestock in Waller County, TX (30.100400, –95.977247) in September and October of 2018 for this study and Gadsden County, FL (30.5563, –84.6479) in July of 2017 as part of an ongoing trapping conducted at a big game preserve (McGregor et al. 2018). All specimens collected from these traps were stored in 95% ethanol at –20.0°C. *Culicoides stellifer* were identified morphologically (Wirth et al. 1985). Voucher specimens of *C. stellifer* collected in Texas were deposited in the Texas A&M University Insect Collection (Voucher 745).

COI Sequencing

Total DNA was extracted from individual *C. stellifer* using a method modified from the Gentra Puregene Kit (Gentra Systems, Inc., D-5500A). PCR reactions targeting a 710-bp region of the *COI* gene were performed to obtain the mitochondrial data (Folmer et al. 1994). Each reaction contained 2.0 µl of DNA, 0.75 µM of each primer, 5.0 µl of 5x reaction buffer, 0.15 µl of Taq, and 16.35 µl

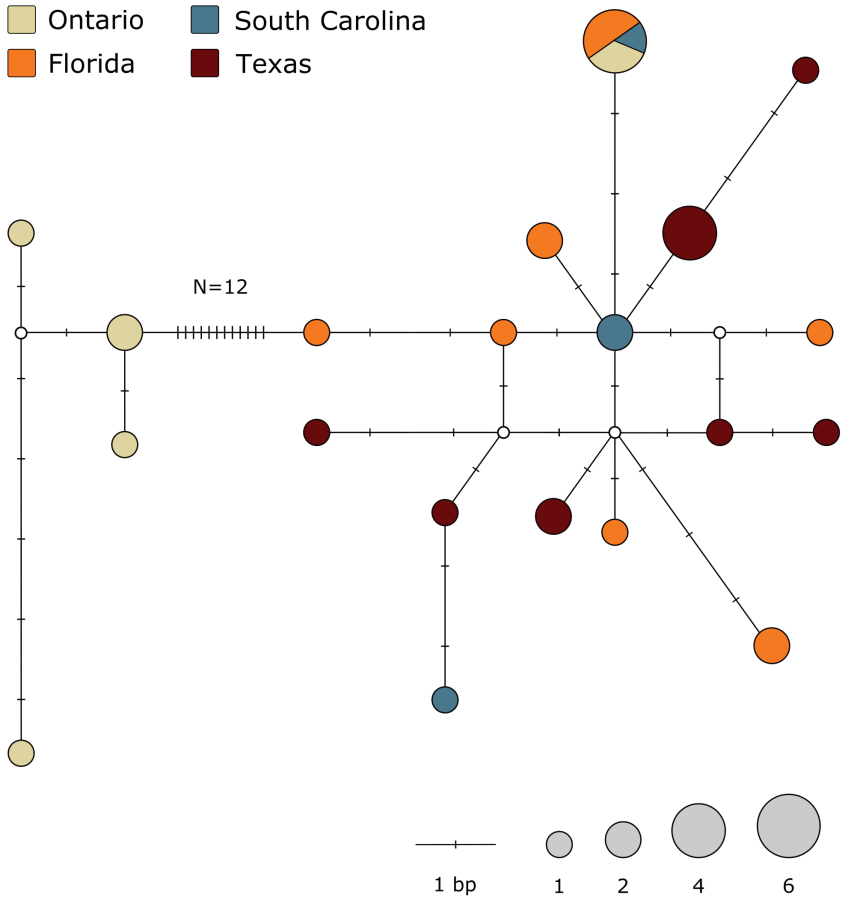

Fig. 1. Median-joining haplotype network diagram of COI mitochondrial sequences from 33 individuals of *C. stellifer* from Ontario, Florida, South Carolina, and Texas. The larger circles indicate more individuals who share the same haplotype, and each tick mark represents 1 base pair (bp) difference. The open circles represent missing haplotypes.

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of deionized water. The thermal cycles used for the amplification of the *COI* gene consisted of 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 45°C for 1.5 min, and 72°C for 2 min, with a final extension step at 72°C for 5 min.

PCR products were cleaned using the EXOSAP-IT protocol (ThermoFisher, 78201.1.ML). The DNA concentrations were measured using a Qubit 3.0 fluorometer and a Qubit dsDNA HS assay kit (Invitrogen, Q33230). Each sample was prepared for sequencing using a BigDye Terminator v3.1 Cycle Sequencer Kit and protocol (Applied Biosystems, 4337454), and sequencing was done using an Applied Biosystems 3500 Genetic Analyzer. Chromatograms produced for each sequence were cleaned and aligned using the program Geneious v. 9.1 (Kearse et al. 2012). Sequences were assigned to species using BLAST search of NCBI GenBank database and accession numbers were assigned, *Culicoides stellifer*: MK585922–MK585938.

Haplotype Network

A mitochondrial haplotype network analysis of *C. stellifer* from the various collection sites was produced using a data set of 11 previously unpublished sequences from Florida and 6 from Texas, as well as previously published sequences; 4 from South Carolina (BOLD, VTGEN09-12, VTGEN012-12, VTGEN013-12, and VTGEN014-12), 7 from Ontario, Canada (BOLD, OPPQO1410-17, and OPPIE438-17), and 5 from Texas (GenBank MH751274–MH751278). After alignment, sequences were trimmed to 388 bp to ensure all sequences contained identical lengths. A median-joining analysis was performed and exported using NETWORK v. 5.0.1.0 (Bandelt et al. 1999), and a final figure was created in Inkscape v. 0.92.4 (Fig. 1). The mean percent divergence between and within populations was calculated in MEGA 7 (Kumar et al. 2016).

Results

The median-joining haplotype analysis of *C. stellifer* included 33 sequences from four geographic locations. The mean percent divergence within these sites varied from 1.0–2.5% and the pairwise mean percent divergence between the sites was 1.1–3.7% (Table 1). The overall genetic diversity seen within populations was equal to the genetic diversity across Florida, Texas, and South Carolina (Fig. 1). The mean percent divergence within Ontario was more than double that of the southern populations at 2.5% (Table 1). Five individuals from Ontario clustered together, divergent from the southern locations; however, two individuals from Ontario had identical haplotypes to individuals from Florida and South Carolina (Fig. 1).

Discussion

Our haplotype network analysis shows genetic similarity across the collections sites in the southeastern United States as well as two individuals from Ontario (Fig. 1). The similarity of these haplotypes and lack of distinct clustering between sites suggest a level of connectivity and gene flow between them. The maximum haplotype divergence between individuals from Florida, Texas, and South Carolina was 2.1%; a level of divergence commonly seen within species (Hebert et al. 2003). In comparison, the maximum sequence divergence of individuals from Ontario was 6.4% (Fig. 1).

If the two individuals from Ontario with haplotypes matching the southeastern localities (ON_1) are compared with the remaining five individuals from Ontario (ON_2), the mean percent divergence between these two groups is 4.2% (Table 2). This also increases how divergent ON_2 is from the southern collection site to 4.5–4.7% (Table 2). Although sequence divergence can vary between taxa, the COI 'barcoding' region is fairly conserved across most insect orders. The level of divergence in the Ontario midges is usually seen between species, not within (Hebert et al. 2003, Arnegard et al. 2014). These data show evidence of a cryptic species closely related to *C. stellifer*.

The lack of clearly defined genetic structure of *C. stellifer* provides evidence that there are little to no barriers of gene flow in the southeast United States; however, this should be examined throughout its range as the population structure of widely dispersed species can vary by region (Kim and Sappington 2004). *Culicoides stellifer* shows the long-distance dispersal patterns that have been observed in other *Culicoides* species, and if this species is indeed involved in the propagation of HD, this lack of population structure could open routes of viral transmission.

Table 1. The mean percent sequence divergence of a 388-bp region of the *COI* gene in *C. stellifer* within and between populations from Florida, Texas, South Carolina, and Ontario

	FL (%)	TX (%)	SC (%)	ON (%)
FL	1.2	–	–	–
TX	1.2	1.1	–	–
SC	1.1	1.1	1.0	–
ON	3.5	3.7	3.5	2.5

Table 2. The mean percent sequence divergence of a 388-bp region of the *COI* gene in *C. stellifer* within and between populations with the population from Ontario split into two groups

	FL (%)	TX (%)	SC (%)	ON_1 (%)	ON_2 (%)
FL	1.2	–	–	–	–
TX	1.2	1.1	–	–	–
SC	1.1	1.1	1.0	–	–
ON_1	1.1	1.4	1.0	0.0	–
ON_2	4.5	4.7	4.5	4.2	1.0

The two individuals that are closely related to the Florida and South Carolina are represented as 'ON_1', and the remaining five individuals that cluster together are represented as 'ON_2'.

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