DISSERTATION

PATHOGEN VECTORS AT THE WILDLIFE-LIVESTOCK INTERFACE: MOLECULAR APPROACHES TO ELUCIDATING *CULICOIDES* (DIPTERA: CERATOPOGONIDAE) BIOLOGY

Submitted by

Matthew W. Hopken

Department of Fish, Wildlife, and Conservation Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2016

Doctoral Committee:

Advisor: Kathryn P. Huyvaert
Co-Advisor: Antoinette Piaggio

Kevin Bestgen
Boris Kondratieff

Copyright by Matthew W. Hopken 2016

All Rights Reserved

ABSTRACT

PATHOGEN VECTORS AT THE WILDLIFE-LIVESTOCK INTERFACE: MOLECULAR APPROACHES TO ELUCIDATING *CULICOIDES* (DIPTERA: CERATOPOGONIDAE) BIOLOGY

Emerging infectious diseases (EIDs) continue to threaten human and animal welfare worldwide. Vector-borne pathogens are particularly concerning because of their ubiquity, pathogenicity, and lack of predictably due to insufficient ecological and evolutionary knowledge regarding vector species. Biting midges in the genus *Culicoides* (Diptera; Ceratopogonidae) have been implicated in the transmission of highly pathogenic viruses and parasites. In North America, the complete transmission cycles of many of these pathogens need further elucidation as outbreaks occur in the absence of known vector species. Further, our knowledge about the evolution and ecology of most *Culicoides* species is limited at best. The focus of research has been morphological identification and distribution of some North American *Culicoides* species, particularly those that are known pathogen vectors, but little is known about the phylogeny, ecology, behavior, and vector competence of many other species, some of which may prove to be important vectors. Ultimately, risk assessments and management of pathogens transmitted by *Culicoides* in North America are limited by this lack of knowledge and these limitations prove to be disastrous. Large and damaging outbreaks of *Culicoides*-transmitted viruses around the globe in the last two decades have raised concerns that North American livestock and wildlife are at

risk. These threats, in conjunction with the lack of knowledge about vector species, led to the current research so that we can more insight into *Culicoides* biology and be proactive in preparing for future outbreaks.

In chapter 1, I argue for more research into the biology of *Culicoides* in North America. In particular, I highlight our lack of information about all potential vector species, draw attention to the issues with the current taxonomic designations, discuss the main pathogens of concern transmitted by *Culicoides*, and then explore outbreaks of bluetongue virus in Europe as a case study illustrating how insufficient knowledge can lead to significant impacts on agriculture and the economy. I also summarize all known and potential vector species in North America to establish recognition of the tenuous nature of data used to incriminate or exclude some *Culicoides* species as vectors. I end with a discussion of the importance of systematics to research and management of vector-borne diseases and make a call for more *Culicoides*-based studies to minimize the biological, ecological, and economic damages caused by the introduction of exotic pathogens.

In Chapter 2, I investigated the phylogentic relationships of the three known and described species in the *C. variipennis* species complex. In North America, two currently recognized vector species, *C. sonorensis* and *C. variipennis,* are thought to drive the transmission of bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV), respectively. Both species, along with a non-vector species *C. occidentalis*, belong to a morphologically similar species complex, the *C. variipennis* complex. The taxonomic history of this complex is convoluted due to the limited number of morphological characters that are species-diagnostic. My objective was to evaluate the current taxonomy of the *C. variipennis* species complex using multi-locus molecular phylogenetics and population genetic approaches in order to understand

evolutionary relationships and historical biogeography within the complex. I determined that molecular data do not support the current taxonomy of the three species in the *C. variipennis* complex. In fact, phylogenetic approaches demonstrated very little resolution at the species level within the *C. variipennis* complex, except for a single clade that represented *C. occidentalis*. I detected weak population genetic structure using two nuclear loci, but this weak structure did not correlate with morphological species identification. I suggest a taxonomic reevaluation of this species complex is sorely needed so that more accurate species identification and distributions can be obtained. Improved species designations lead to more precise inferences about ecology and evolution of vector species. Through this taxonomic demystification, we can better grasp sylvatic transmission cycles and prepare for outbreaks of both domestic and foreign pathogenic viruses.

In Chapter 3, I investigated host choice of *Culicoides* species collected in North America using DNA-based bloodmeal analyses. In North America, we currently lack the ability to understand transmission networks and assess outbreak risk for Culicoides-borne pathogens as our knowledge base of trophic ecology is meager at best. Knowing the diversity of vertebrate hosts for *Culicoides* can help identify susceptible species and pathogen reservoirs, and can lead to the detection of new vector species. The objective of this study was to identify the diversity of *Culicoides* hosts in North America. I sequenced two vertebrate mitochondrial genes (cytochrome oxidase I and cytochrome B) from blood-engorged *Culicoides*. I detected the mitochondrial DNA of 12 host species from five different *Culicoides* species and the *C. variipenis* species complex. The hosts included both mammals and birds. I documented new host records for some of the *Culicoides* species collected. The majority of the mammalian hosts were large ungulate species but I also detected a lagomorph and a carnivore. The bird species that were detected

included House Finch and Emu, the latter is evidence that the species in the *C. variipennis* species complex are not strictly mammalophilic. These results demonstrate that *Culicoides* species will feed on multiple classes of vertebrates and may be more opportunistic in regards to host choice than previously thought.

Throughout the dissertation, I have drawn attention to the areas for which we lack the most knowledge regarding North American *Culicoides*, illuminated the evolutionary relationships and taxonomic discrepancies in the *C. variipennis* complex, and improved our understand of *Culicoides* host choice. These are the first steps towards developing an improved understanding of *Culicoides* biology, in general, and the sylvatic cycles of *Culicoides*-borne pathogens, in particular. My research has also raised other important questions that deserve more attention so that we can better grasp the impacts that anthropogenic change has on *Culicoides* ecology, evolution, and pathogen transmission. As we increase our knowledge about this important group of insects, the ability to predict, prepare, and mitigate the economic and ecological damage from disease outbreaks will be greatly improved.

ACKNOWLEDGMENTS

I thank numerous individuals that helped make this project happen. K. Huyvaert, A. Piaggio, B. Kondratieff, and K. Bestgen served as my committee members, provided helpful scientific discussion, and commented on earlier drafts of this manuscript. D. Griffin, N. Breuner, D. Wostenberg, J. Carlson, M. Selleck, M. Lutman, S. Vigil, SCWDS, E. Maki, USDA-ARS, M. Becker, L. Foil, B. Ryan, Lake County Vector Control Agency and numerous other vector control agencies helped with sample collection, identification of specimens, or molecular lab work. I also thank C. Myrick (CSU) and the many landowners that allowed me to trap on their property. S. Shriner, S. Elmore, B. Mosher, K. Williams, and M. Robeson provided input on study design, presentation, analysis, and moral support. I appreciate B. Grogan's generosity with his time, donation of slide-mounted specimens, and insight into the biology of *Culicoid*es. There were also numerous silent champions without whom this research would be lacking. Finally, I would like to thank my son, Hiro, for being a source of unending joy and inspiration.

TABLE OF CONTENTS

Abstract ii

Acknowledgements vi

Chapter 1: North American *Culicoides* (Diptera: Ceratopogonidae): taxonomy, phylogeny, vector potential, and the risks of knowing too little 1

Introduction 1

Culicoides and pathogen transmission 5

Threat of *Culicoides*-transmitted pathogens to North America: Europe as a case study 8

State of knowledge: Natural populations of North American *Culicoides* as vectors 11

Knowledge gaps and potential implications 20

Table 24

Literature Cited 25

Chapter 2: Multi-locus phylogeny reveals extensive taxonomic uncertainty in the *Culicoides variipennis* species complex (Diptera: Ceratopogonidae) 46

Introduction 46

Materials and Methods 51

Results 58

Discussion 62

Tables 73

Figures 74

Literature Cited 82

Chapter 3: Picky eaters are rare: DNA-based bloodmeal analysis of the *Culicoides variipennis* species complex (Diptera: Ceratopogonidae) 111

Introduction 111

Materials and Methods 113

Results 115

Discussion 116

Table 122

Figure 123

Literature Cited 124

Appendices 132

CHAPTER 1

North American *Culicoides* (Diptera: Ceratopogonidae): taxonomy, phylogeny, vector potential, and the risks of knowing too little.

Introduction

Emerging infectious diseases (EIDs) are inextricably linked with unprecedented global changes in climate, human demographics, urbanization, and globalization (Bram et al., 2002; Jones et al., 2008; Sutherst, 2004). Intensified human encroachment on natural habitats, expanding travel and commerce, and changes in species' distributions induce shifts in pathogen home ranges and increase contact with naïve hosts (Harrus and Baneth, 2005). These largely anthropogenic impacts to co-evolutionary networks among hosts, pathogens, and vectors have been linked to large-scale disease outbreaks (Jackson and Tinsley, 2005). When pathogens infect new populations, either by direct human introduction or unassisted range expansions (e.g., as a result of climate change), the consequences can be devastating to the health and welfare of humans (Weaver and Barrett, 2004), domestic animals (Mellor and Wittmann, 2002), and native wildlife (Daszak et al., 2000).

Arthropod-borne pathogens are particularly concerning because they encompass more than a quarter of the pathogens responsible for recent EIDs (Jones et al., 2008) and are expected to be highly virulent (Ewald, 1995) leading some to label pathogen-infected vectors as "…the most dangerous animals on Earth" (Besansky et al., 2003). Yet we lack fundamental knowledge of many vector-pathogen systems particularly concerning vector taxonomy and species identification (Tabachnick, 2004; Tabachnick and Black, 1995). The significance of a

comprehensive knowledge base regarding vector biology cannot be overstated given the role of vectors in pathogen transmission and that many vectors are sites of arboviral reassortment, a process which can drive evolutionary change in virulence (Samal et al., 1987). The inability to precisely identify many vector species, because of their small size, fragility, limited diagnostic morphological characters, and the presence of cryptic species, has severely impeded research and surveillance of arthropod-borne animal pathogens (Carpenter et al., 2009; Harrup et al., 2015). Considering that morphologically cryptic species obscure true biogeographic patterns, robust species identification and concomitant taxonomies that reflect underlying phylogenetic history translate into more accurate disease risk assessments (Harbach, 2004; Pagès et al., 2009).

Biting midges in the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) are extremely small (2-3 mm) and are some of the most abundant blood feeding flies (Mellor et al., 2000). More than 1400 extant species have been described worldwide (Borkent, 2015) with a small percentage implicated as vectors of highly pathogenic viruses, Protozoa, and Nematoda (Meinswinkel et al., 1994). *Culicoides* are generally globally distributed but are absent from Antarctica and New Zealand (Meinswinkel et al., 1994; Mellor et al., 2000). They occur in diverse habitats, from the tropics to the tundra, and can be found at elevations extending from sea level to 4000 m (Mellor et al., 2000). Over 50 arboviruses have been isolated from *Culicoides*, 45% of which have not been detected in other arthropods (Mellor et al., 2000). The pathogens of most concern are bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), African horse sickness virus (AHSV), and Schmallenberg virus (SBV), together these cause morbidity and mortality of thousands of wild and domestic ungulates worldwide each year leading to significant economic repercussions. In the Netherlands, for example, the direct and indirect costs to agriculture of the BTV serotype 8 (BTV-8) outbreak in 2006 exceeded one billion U.S. dollars

(Hoogendam, 2007; Wilson and Mellor, 2009). Further, the impact of EHDV on global and local economies can be large considering the multi-million dollar losses from cattle and dairy production, potential losses to the U.S. deer farming industry whose current economic impacts are worth more than $800 million, and the resulting lost revenue for states and local economies due to hunting restrictions (Anderson et al., 2007; Grado et al., 2007; Kedmi et al., 2010; Montana Fish, 2011). Finally, disease-related livestock trade restrictions between BTV and BTV-free countries results in millions of dollars of lost revenue, particularly for the U.S. (Hoar et al., 2003; Tabachnick, 1996).

The knowledge base about the biology of the majority of *Culicoides* species is meager as most research has focused solely on a handful of species that have been implicated as vectors of pathogens of concern (Harrup et al., 2015; Purse et al., 2015). The majority of our knowledge in North America concerns the *C. variipennis* (Coquillet) species complex (hereafter *C. variipennis* complex) which includes three morphologically similar species, one of which, *C. sonorensis* Wirth and Jones, is a primary vector of BTV and EHDV; but even here there are large gaps. At the most basic level, the phylogenetic relationships among North American *Culicoides* spp. are unresolved, detailed estimates of species' geographic ranges are lacking, the breadth of host choice for each species is unknown, and the vector competence of many species has never been investigated. This latter information is important because species belonging to the *C. variipennis* complex have been absent or rare in light trap catches at a number of EHDV outbreak sites in the U.S. (M. Lutman, personal communication; Smith et al., 1996b), and the viruses have been detected in or isolated from a number of non-*variipennis* species suggesting that other transmission cycles may exist (e.g., Becker et al., 2010). Even more troubling is that BTV-8, the serotype that caused the deaths of millions of sheep in Europe, has been detected in the

Caribbean, a part of the native range of *C. insignis* Lutz (Anderson et al., 2007; Wilson et al., 2009). *Culicoides insignis* is one of the most abundant species in the Caribbean and Florida, is associated with livestock, and has been shown to transmit multiple BTV serotypes (Blanton and Wirth, 1979; Tanya et al., 1992) to livestock. Both *C. sonorensis* and *C. insignis* are sympatric in northern Florida, southern Alabama, and Georgia (Borkent and Grogan, 2009; Kramer et al., 1985a, b; Vigil et al., 2014). The distributional patterns of the two vector species mirror that of the European outbreaks where the African subtropical species *C. imicola* overlapped with competent northern European *Culicoides* species (Carpenter et al., 2009). This led to transfer of the virus into northern Europe which led to widespread outbreaks and severe economic losses. The range overlap of *C. sonorensis* and *C. insignis* is concerning because wind-dispersed BTV infected *Culicoides* from the Caribbean were the most likely source of introduction of BTV-2 in Florida (Sellers and Maarouf, 1989). Thus, if *C. insignis* is a competent vector of BTV-8 and infected vectors, or infected ungulates, are transported to Florida, then BTV-8 or other potential pathogenic serotypes or viruses could easily be transferred to latent vector species and impact local cervid populations, or spread rapidly throughout the country (Maclachlan and Mayo, 2013).

In light of recent *Culicoides*-borne viral outbreaks in North America and abroad (e.g., Carpenter et al., 2009; Koenraadt et al., 2014; ProMED-mail, 2012), and the detection of exotic viral serotypes in North America (Allison et al., 2010), the goals of this paper are threefold: 1) to review the current state of scientific knowledge about *Culicoides* in North America with an emphasis on the systematics and vector potential of species not currently recognized as vectors; 2) to elucidate knowledge gaps that impede disease research, surveillance, and response activities, particularly for BTV and EHDV; and 3) to outline immediate and future *Culicoides* research needs in North America in anticipation of potential outbreaks with the goal of

mitigating impacts on livestock and wildlife populations. Herein, we reiterate and expand on previous calls to action for vector-focused ecological and evolutionary research (e.g., Tabachnick and Black 1995) with particular emphasis on *Culicoides* in North America. We further draw upon two recent reviews about the lack of knowledge globally regarding *Culicoides* and pathogen transmission (Harrup et al., 2015; Purse et al., 2015) to demonstrate that this global issue has serious repercussions specific to North American agriculture and wildlife. We discuss how potential impacts have not previously the received the attention that is necessary to mitigate the effects of future outbreaks.

Culicoides and pathogen transmission

Worldwide, *Culicoides* transmit 113 known pathogens, some of which are highly pathogenic to animals and humans (Table 1; Borkent, 2005). Oropouche virus (Bunyaviridae) and *Masonella ozzardi* Manson (Nematoda: Onchocercidae), for example, are the two main human pathogens transmitted by *Culicoides* in North and South America. But the greatest concern revolves around a number of serious animal pathogens such as the arboviruses ASHV, BTV, EHDV, SBV, and the avian blood parasite *Haemoproteus meleagridis* Levine (Haemosprodia: Haemoproteidae).

Arboviruses within the genus *Orbivirus* (Reoviridae) are classified by double stranded RNA, unique protein structure, and predominant transmission by arthropod vectors (Gould and Hyatt, 1994; MacLachlan and Guthrie, 2010). The genus includes a number of economically important animal pathogens including BTV, EHDV, and African horse sickness virus (AHSV). The geographic distribution of BTV and EHDV corresponds to that of the *Culicoides* vectors. Both viruses occur in a global band between latitudes 40°N and 35°S, with occasional excursions as far north as 50°N (Mellor et al., 2000; Wilson and Mellor, 2009). Outbreaks of disease occur

in late summer to early fall, coinciding with the highest vector densities, and transmission halts at the onset of frost when vectors die (Mellor et al., 2000). The overwintering mechanisms for both viruses are unknown and little evidence exists for transovarial transmission (Osborne et al., 2015; White et al., 2005; Wilson et al., 2008).

Both BTV and EHDV cause periodic outbreaks within their enzootic ranges, but also invade new areas leading to significant mortality in affected wildlife and livestock populations (Hourrigan and Klingsporn, 1975). Two, not mutually exclusive, hypotheses exist explaining the transfer of *Culicoides*-transmitted viruses between regions: 1) movement of *Culicoides* either by wind or climate-induced range shifts (Maclachlan and Mayo, 2013; Purse et al., 2005; Sellers and Maarouf, 1989); or 2) movement of infected livestock by humans (Tabachnick, 2010). *Culicoides* are known to disperse many kilometers on strong winds (Mellor et al., 2000) and evidence supporting the climate change hypothesis has been summarized elsewhere (Maclachlan and Mayo, 2013; Purse et al., 2005). However, the fact that each month millions of livestock are transported across international borders, and some imported animals that are potential carriers (i.e., exotic zoo animals) are not tested for either virus, has led to support for the latter hypothesis (Bram et al., 2002; Tabachnick, 2010). A third hypothesis is that EHDV and BTV are transmitted between regions and maintained by wildlife populations, a process that has been suggested as playing a role in recent European outbreaks (Falconi et al., 2011; García-Bocanegra et al., 2011; Ruiz-Fons et al., 2014).

Bluetongue virus consists of 25 serotypes isolated from all continents except Antarctica (MacLachlan and Guthrie, 2010). All ruminant species are susceptible to infection with BTV with clinical symptoms typically developing in domestic sheep (*Ovis aries* L.) and white-tailed deer (*Odocoileus virginianus* (Douglas)), with a number of other ruminants [e.g. bighorn sheep,

(*Ovis canadensis* Shaw); domestic cattle (*Bos taurus* L.)] harboring the virus in their blood for prolonged periods but are asymptomatic (Maclachlan et al., 2009; Noon et al., 2002). Serotypes that are enzootic in North America are BTV-2, 10, 11, 13, and 17; all of these serotypes can cause periodic outbreaks, mostly in domestic sheep and white-tailed deer. For example, a BTV-17 epizootic in Wyoming in 2008 caused significant domestic sheep mortality that reached 12% on some ranches (Miller et al., 2010b). The prevalence of BTV in the U.S is higher in western and southern states and considerably lower in northeastern states, given they are outside the expected range of the only known vector, *C. sonorensis* (Ostlund et al., 2004; Tabachnick, 1996).

The main threats to U.S. agriculture and wildlife, however, come from exotic serotypes of BTV because known competent vectors are present (e.g., *C. sonorensis* and *C. insignis*) (Johnson et al., 2006; Tanya et al., 1992), susceptible livestock (sheep) and wildlife (white-tailed deer) hosts exist, and exotic serotypes are known to be highly pathogenic when invading countries with naïve hosts. For example, from 1998-2006, Europe experienced major epizootics of exotic pathogenic BTV serotypes that affected animals in 12 countries in the Mediterranean region and then extended 800 km further north than previously recorded (Purse et al., 2005). The most pathogenic serotype, BTV-8, not only caused clinical symptoms and killed more than 1 million domestic sheep, but the outbreak also caused morbidity in cattle which are usually asymptomatic (Darpel et al., 2007). During this outbreak some countries lost up to 15% of their domestic sheep (Enserink, 2008).

EHDV currently includes seven serotypes worldwide (Savini et al., 2011). The enzootic serotypes in North America, EHDV-1 and -2, lack any geographic structure which suggests that these pathogens move freely across the landscape (Mecham et al., 2003). EHDV disease outbreaks occur in two to three year cycles in endemic areas and major epizootics happen every

eight to ten years (Nettles et al., 1992; Stallknecht and Howerth, 2004). All ruminants are susceptible to infection with EHDV but only a few taxa develop clinical symptoms. The species with the greatest morbidity and mortality rates from EHDV is white-tailed deer; however, certain strains of EHDV-1 and -2 can cause disease in bighorn sheep, mule deer (*Odocoileus hemionus* Rafinesque), and pronghorn (*Antilocarpa Americana* (Ord)) (Noon et al., 2002; Work et al., 1992). The average mortality for infected white-tailed deer is 20% (Gaydos et al., 2004); thus this disease is of major concern for wildlife managers, conservation biologists, and cervid livestock producers. In 2011 and 2012, major epizootics of EHDV killed thousands of white-tailed deer in the Northern Plains and northeastern U.S. with population mortalities reaching 90% at times (ProMED-mail, 2012). The only livestock species that is known to be susceptible to EHDV is cattle, which displays clinical symptoms when infected with EHDV-2 (Ibaraki strain), and serotypes EHDV-6 and -7. Historically, EHDV-6 and -7 were considered non-pathogenic in livestock but recent outbreaks in the Near East and North Africa caused disease in cattle and significant losses to dairy production (Savini et al., 2011).

Threat of *Culicoides*-transmitted pathogens to North America: Europe as a case study

Europe experienced a prolonged period of outbreaks of multiple exotic BTV serotypes that culminated in the 2006 outbreaks of the highly pathogenic BTV-8 (Carpenter et al., 2009; Wilson and Mellor, 2009). During these outbreaks, BTV moved into areas that were previously thought to be BTV-free because no competent vectors were believed to be present. The initial hypothesis of viral invasion was that the North African vector species, *C. imicola* (Kieffer), which made occasional incursions into Mediterranean Europe, became established in Europe due to climactic changes that facilitated long-term vector survival (Purse et al., 2005). Despite ongoing pathogen transmission, however, northern countries on the leading edge of the outbreaks

failed to detect *C. imicola* (Carpenter et al., 2009). Native *Culicoides* species were investigated for viral infections and multiple species from two species complexes, *C. obsoletus* Meigen and *C. pulicaris* (Linnaeus), were implicated (Hoffmann et al., 2009). Evidence demonstrated that the range of *C. imicola* did expand and that this species is now established in European countries bordering the Mediterranean (Purse et al., 2005). The expanded range of *C. imicola* overlapped with the ranges of native *Culicoides* spp., some of which were competent vectors that subsequently spread the novel pathogen into new areas with naïve and susceptible ungulate populations (Caracappa et al., 2003; Dijkstra et al., 2008; Meiswinkel et al., 2007).

The possibility that BTV could invade northern Europe and cause major outbreaks was identified as early as 1993 based on increases in farming breeds of sheep that were highly susceptible to BTV infections (Carpenter et al., 2009). However, very few resources were directed at increasing the knowledge of BTV epidemiology and vector biology (Carpenter et al., 2009; Wilson and Mellor, 2009). *Culicoides* were often overlooked as research topics because they are small and difficult to identify morphologically and Europe had few *Culicoides* specialists. But following the initial outbreaks in the early 2000's, molecular phylogenetic studies were initiated to assist with elucidation of vector taxonomy and identification with the goal of determining the mechanisms underlying the geographic variability of *Culicoides* spp. susceptibility to BTV infection (Carpenter et al., 2009). Through multiple phylogenetic studies using both nuclear and mitochondrial DNA, evidence surfaced that morphology-based taxonomy did not always correlate with phylogeny. The *C. obsoletus* and *C. pulicaris* species complexes, and multiple species within those complexes, were identified as polyphyletic, consisting of divergent morphologically cryptic species (Pagès et al., 2009; Schwenkenbecher et al., 2009). These studies highlight the importance of coupling molecular phylogenetics with morphological

taxonomy to assist with vector species identification, enhancing vector-borne disease research and management (Harrup et al., 2015). Due to the discovery of divergent cryptic species, BTV research and surveillance have been streamlined in Europe by focusing on the most likely vector species and the regions that contain these species. But, despite this work, little is still known about *Culicoides* taxonomy and biogeography in most parts of Europe (Wenk et al., 2012). A recently discovered *Culicoides*-transmitted pathogen with an unknown origin, Schmallenberg virus, is currently impacting European livestock and wild ungulate populations by causing fetal deformations and stillbirths (Linden et al., 2012; Tarlinton et al., 2012). This substantiates the case that we know very little about these vector-borne pathogen systems and that knowledge deficits pose significant threats to wildlife and livestock health.

A similar scenario currently exists in North America and, unfortunately, the U.S. does not have a comprehensive nationwide surveillance program (accounting for both vertebrate hosts and vectors) for BTV or EHDV (Gibbs et al., 2008). Few *Culicoides* taxonomists currently work in North America and relatively little has been invested in training the next generation of taxonomic entomologists (Leather, 2009; Packer et al., 2009), which is a common theme worldwide for all organisms and has been coined the "taxonomic impediment" (Wheeler et al., 2004) as the importance of taxonomy has been down weighted when compared to other disciplinces. A significant amount of research has been conducted into the molecular biology of *C. sonorensis* and has mostly focused on molecular mechanisms of viral dissemination and surveillance-based documentation of occurrence and local abundance (e.g., Campbell et al., 2005; Schmidtmann et al., 2011), but there has been little focus on the biogeography of the genus, identification of other potential vector species, and elucidation of the evolutionary relationships among the endemic Nearctic *Culicoides*. The latter information would contribute to global research because

Culicoides subgenera have a Holarctic distribution and the global phylogeny is unknown as most studies focus on small geographic areas (Harrup et al., 2015; Meinswinkel et al., 1994).

State of knowledge: Natural populations of North American *Culicoides* as vectors

More than 300 *Culicoides* species have been described in North America with more than 150 species occurring north of Mexico (Borkent and Grogan, 2009). Approximately 16% of these species (n = 24) have been implicated in the transmission of pathogens; most of these are associated with arboviruses (Table 1).

The main vectors of concern in the U.S.: Culicoides variipennis *species complex*

The *C. variipennis* complex is currently classified as belonging to the subgenus *Monoculicoides* Khalaf (Khalaf, 1954). Only two other member species of this subgenus are known to occur in North America, *C. gigas* Root and Hoffman (Wirth and Jones, 1957) and *C. grandensis* Grogan and Phillips (Grogan Jr. and Phillips, 2008). Holbrook et al. (2000) summarized the taxonomic history of the *C. variipennis* complex which is briefly paraphrased here. *Culicoides variipennis* was originally recognized as a single species with five subspecies: *C. v. albertensis* Wirth and Jones, *C. v. australis* Wirth and Jones, *C. v. occidentalis* Wirth and Jones, *C. v. sonorensis*, and *C. v. variipennis* (Wirth and Jones, 1957). Atchley (1967) synonymized *australis* with *sonorensis*. *Culicoides variipennis* and *C. occidentalis* were later recognized as distinct species with the remaining taxa considered subspecies of *C. occidentalis* (Downes, 1978). Considering the difficulties of *Culicoides* morphometrics, Hensleigh and Atchley (1977) suggested that morphology alone cannot be used to taxonomically separate the species within the *C. variipennis* complex and that electrophoretic (allozyme) techniques should

be utilized. Wirth and Morris (1985) noted that genetic data were required to further assist taxonomic ranking within this group.

Based on allozyme allele frequencies, three putative subspecies were recognized: *C. v. occidentalis, C. v. sonorensis*, and *C. v. variipennis* (Tabachnick, 1990, 1992). Additional work confirmed the taxonomic uniqueness of each subspecies (Holbrook and Tabachnick, 1995; Holbrook et al., 1996). Allozyme, morphological, biogeographical, and ecological data were ultimately combined and suggested that each of the three subspecies should be elevated to species status and synonymized *C. v. albertensis* with *C. sonorensis* (Holbrook et al., 2000). Morphology-based species identification within the *C. variipennis* complex is challenging as it is based on minor characters that require time-consuming dissection and slide mounting, and the diagnostic characters are sex-specific (Holbrook et al., 2000). Adding to the complexity is intraspecific variation in many of the species-diagnostic morphological traits which sometimes are lacking in different parts of the geographic ranges (Holbrook et al., 2000). Multi-locus allozyme analyses using differences in allele frequencies have allowed differentiation of these three species, but no species-diagnostic alleles have been observed (Tabachnick, 1992). Along with an inability to precisely identify these three species (recognition is mostly based on geography and breeding habitat), range-wide intraspecific variability exists in susceptibility to viral infection (Tabachnick, 2004), which suggests the possibility that morphologically cryptic taxa exist or environmental influences on vectorial capacity are quite strong, as has been shown with Palearctic *Culicoides* in Europe (Harbach, 2004; Pagès et al., 2009; Purse et al., 2015).

The known distribution of the *C. variipennis* species complex in North America is broad, including regions of Canada, most of the United States, and extends as far south as Mexico City, Mexico (Holbrook et al., 2000; Huerta et al., 2012; Wirth and Jones, 1957), but the exact ranges

of each of the three species are unknown. Most authors agree that *C. variipennis* is generally distributed in eastern North America, *C. sonorensis* is distributed in southern and western North America, and *C. occidentalis* is found in extreme western North America. All three species have sizeable regions of overlap, particularly in the central, southeastern, and southwestern U.S. (Holbrook et al., 2000; Schmidtmann et al., 2011).

The *C. variipennis* complex has been the focus of vector research because *C. sonorensis* is known to transmit multiple pathogens, and this species is more amenable to rearing in captivity than most other *Culicoides* species. (Holbrook et al., 2000; Tanya et al., 1992). *Culicoides sonorensis* is currently recognized as the main vector of BTV, a known vector of EHDV, and its competence as a vector has been demonstrated for African horse sickness virus, Akabane virus, vesicular stomatitis virus, and Schmallenberg virus (Table 1; Boorman et al., 1975; Jennings and Mellor, 1989; Nettles et al., 1992; Pérez de León and Tabachnick, 2006; Tabachnick and Holbrook, 1992; Veronesi et al., 2013). The other two species in the complex, *Culicoides variipennis* and *C. occidentalis* Wirth and Jones, have not yet been implicated in the transmission of any arboviruses.

Other known or potential North American vectors of BTV and EHDV

The current paradigm in the transmission of BTV and EHDV in North America suggests that *C. sonorensis* is the main and most important vector (Tabachnick, 2010). However, as previously mentioned there have been instances where *C. sonorensis* has been absent from light trap catches during EHDV epizootics, thus there is the potential that other *Culicoides* species play roles in the transmission and maintenance of these viruses. In Europe, multiple phylogenetically distant species are implicated in the transmission of BTV which points towards the possibility of multiple, alternate transmission cycles in North America (Purse et al., 2015).

This section will highlight other species of *Culicoides* in North America that have been identified either as competent vectors, e.g., *C. insignis*, or others from which data from natural populations has pointed towards vector potential (Table 1). We discuss the distribution and taxonomy of these species as well as data that highlights the need for further research to determine vector competence and vectorial capacity.

The other confirmed vector of BTV in North America is *C. insignis* Lutz (Tanya et al., 1992). *Culicoides insignis* is one of two Nearctic species of the subgenus *Hoffmania* Fox (Borkent and Grogan, 2009). The distribution of *C. insignis* includes Alabama and Georgia, south throughout Florida, the Caribbean, and Central America (Borkent and Grogan, 2009). Fortunately, *C. insignis* is quite easy to identify from wing patterns which simplifies surveillance (Blanton and Wirth, 1979). As previously mentioned, the distribution of this species overlaps with that of *C. sonorensis* which points to a potential introduction route for exotic BTV serotypes from the Caribbean.

A number of other North American species have been suspected of playing roles in the transmission of BTV and EHDV. *Culicoides venustus* Hoffman is in the same subgenus, *Hoffmania*, as *C. insignis* and is distributed from Nova Scotia in the north, south to Louisiana and Florida, and from the Atlantic coast west to Nebraska (Borkent and Grogan, 2009). Jones et al. (1983) were able to infect *C. venustus* with both BTV and EHDV, albeit at extremely low rates. They concluded that *C. venustus* is not an efficient vector. However, they noted that these results were based on a single population and that vector species, such as *C. sonorensis*, often vary in susceptibility to infection across their geographic ranges. These low infection rates in the lab should not preclude species from consideration as potential vectors. Field caught vectors often display low infection rates; for example, only 2.4% of *Culicoides* trap catches collected in

Germany were positive for BTV-8 during the 2007-2008 outbreak suggesting that low infection rates can still drive epizootics (Hoffmann et al., 2009). However, the relatively low abundance of this species may still be a limiting factor for vectorial capacity (Blanton and Wirth, 1979).

Culicoides stellifer (Coquillett) is currently placed in the subgenus *Oecata* and is a common species occurring in Canada, and all U.S. states except Washington and Oregon, and Mexico (Table 1; Borkent and Grogan, 2009; Smith et al., 1996b). This species has been associated with livestock and captive deer, and identified as one of the most abundant species at EHDV outbreak sites where *C. sonorensis* was found to be rare or absent (Smith and Stallknecht, 1996). Additionally, Mullen et al. (1985a) demonstrated that, while *C. stellifer* is difficult to rear in the laboratory, it can maintain an infection with BTV-7 for up to 14 days; but neither BTV nor EHDV have been detected in wild-caught *C. stellifer* to date (Becker et al., 2010; Greiner et al., 1984a; Wiesers-Chimpf et al., 1993). Interestingly, Swanson (2012) found 4 divergent cytochrome-c oxidase subunit I (COI) lineages in *C. stellifer* collected in South Carolina, and these lineages were polyphyletic. Considering the broad range of this species, these findings open the possibility for the presence of cryptic species. *Culicoides stellifer*'s ability to maintain a BTV infection in lab settings, its high abundance at outbreak sites, affinity for large ungulates, and the presence of cryptic lineages hints that *C. stellifer* may play a role in BTV and/or EHDV transmission and could be studied further.

Culicoides debilipalpis Lutz belongs to the subgenus *Haematomydium* and is distributed from Maryland, west to Nebraska south to Florida and extends to Argentina (Borkent and Grogan, 2009; Spinelli and Ronderos, 1997). The taxonomy of this species has been complicated (Huerta et al., 2012). In North America, this species was originally identified as *C. debilipalpis* (Blanton and Wirth, 1979) which was subsequently considered to be a junior synonym of *C.*

lahillei Iches (reviewed in Spinelli and Ronderos, 1997). Spinelli and Ronderos (1997) compared specimens from Argentina and resurrected the species *C. debilipalpis* and determined that the North American individuals are actually *C. debilipalpis* and not *C. lahillei*. The alternating taxonomy resulted in two studies in the U.S. using different species names, one that tested the susceptibility to infection of *C. debilipaplis* with BTV (Mullen et al., 1985a), and another that tested the susceptibility of *C. lahillei* (which was actually *C. debilipalpis*) to infection with EHDV (Smith et al., 1996a). These studies showed that *C. debillipalpis* could maintain BTV and EHDV infections, although at very low levels (Mullen et al., 1985a; Smith et al., 1996a). *Culicoides debilipalpis* was one of the most abundant species at two EHDV outbreak sites and > 20,000 individuals have been collected from one deer in a single morning (Smith and Stallknecht, 1996; Smith et al., 1996a; Smith et al., 1996b). Thus, susceptibility to infection, abundance, and preference for large ungulates suggest that this species may play a role in the transmission cycles of both BTV and EHDV and thus requires further study.

Species in the subgenus *Avaritia* Fox have been implicated in the transmission of BTV in Europe, Central America, and the Caribbean. *Culicoides obsoletus* (Meigen) is a confirmed vector and *C. chiopterus* (Meigen) is a potential vector in Europe (Carpenter et al., 2008; Dijkstra et al., 2008). But despite the species' Holarctic distributions and the high abundance of *C. obsoletus* in some regions (Jamnback and Wirth, 1963), they have not yet been confirmed as vectors in North America (Harrup et al., 2015). One potential reason for the lack of interest in evaluating *C. obsoletus* and *C. chiopterus* in North America is because their highest abundances are in eastern North America where BTV infections are rarely detected (Tabachnick, 1996). *Culicoides pusillus* Lutz is a known vector in Central America and the Caribbean (Mo et al., 1994) but vector competence or vectorial capacity have not been tested where the species exists

in southern Florida. The presence in North America of these species that are known vectors of BTV in other areas of the world begs the question, what is the possibility that these widely distributed species are potential arbovirus vectors in North America, and, in particular, what is the risk that is posed by these species if a pathogenic virus, such as BTV-8 or Schmallenberg virus, is introduced into North America?

Other *Culicoides* species in North America have been suspected of playing roles in the transmission of BTV and EHDV based on virus isolation, positive PCR tests for viral RNA, their affinity for large mammals, and/or abundance and presence at sites of ongoing or recent outbreaks. Bluetongue virus or EHDV have been detected with PCR or isolated from *C.*(*Culicoides*) *cockerellii* (Coquillett), *C.* (*Beltranmyia*) *crepuscularis* (Malloch), *C.*(*Oecata*) *furens* (Poey), *C.* (*Diphaomyia*) *haematopotus* Malloch, (Becker et al., 2010; Kramer et al., 1990). Other species have been detected in high numbers around seropositive ungulates, or in areas with ongoing outbreaks. These species are *C. mohave* Wirth, not placed into any existing subgenus (Rosenstock et al., 2003), *C.* (*Avaritia*) *boydi* Wirth and Mullens (Wirth and Mullens, 1992), *C.* (*Silvaticulicoides*) *biguttatus* (Coquillett), *C.* (*Haematomydium*) *paraensis* (Goeldi), and species in the subgenus *Selfia* which have morphologically indistinguishable females (Mullen et al., 1985b; Mullens and Dada, 1992a; Schmidtmann et al., 1980).

Other pathogens of concern

Culicoides spp. could likely play a role in transmission of zoonotic pathogens as multiple arboviruses have been either isolated from or detected in North American *Culicoides* species (Carpenter et al., 2013). West Nile virus (WNV) was introduced into North America in 1999, belongs to the Flaviviridae, and is known to cause encephalitis in humans and horses which, in the worst cases, can lead to death of immunocompromised individuals (Lanciotti et al., 1999;

Petersen and Hayes, 2008). Birds have been identified as amplification hosts in the sylvatic cycle with *Culex* mosquitoes (Diptera; Culicidae) transmitting the virus (Kilpatrick, 2011). In North America, WNV has also been detected in pools of *C. biguttatus*, *C. (Amossovia) arboricola* Glukhova, *C. sonorensis*, and *C. stellifer* suggesting that non-mosquito biting insects could influence virus transmission (Naugle et al., 2004; Sabio et al., 2006). Oropouche virus is a major zoonotic arbovirus from South and Central America in the family Bunyaviridae that causes febrile illness in humans (Anderson et al., 1961; Pinheiro et al., 1982; Pinheiro et al., 1981b). The main vector is *C. (Haematomydium) paraensis* (Goeldi) which occurs from the northeastern and southern U.S. to Argentina (Pinheiro et al., 1981b); however, vector competence and transmission have only been investigated in *C. paraensis* populations south of the U.S. Vesicular stomatitis virus (VSV) is in the family Rhabdoviridae and mainly infects cattle, horses, and swine, leading to illness which can inflict economic damage on livestock producers (Osborne et al., 2015). Many other mammals, including humans, can be infected with impacts ranging from asymptomatic to resembling a mild flu (Osborne et al., 2015). Multiple insect groups including mosquitoes (Culicidae), black flies (Simuliidae), and sand flies (Psychodidae) have been implicated in transmission (Bergold et al., 1968; Mead et al., 2000; Tosh et al., 1971). However, the virus has been isolated from *C. sonorensis, C. stellifer,* and *C.* (*Selfia*) sp., and *C. sonorensis* has transmitted VSV in laboratory settings (Drolet et al., 2005; Kramer et al., 1990; Pérez de León et al., 2006; Pérez de León and Tabachnick, 2006; Walton et al., 1987).

Many non-viral pathogens are associated with *Culicoides* spp. that occur in North America and more complete reviews of these can be found elsewhere (Borkent, 2005; Table 1; e.g., Linley, 1985; Valkiunas, 2004). We provide a few examples to demonstrate the breadth of

pathogens that potentially can be transmitted by North American *Culicoides* species and could have economic, health, and animal welfare repercussions.

Multiple taxa of filarial worms (Nematoda) and protozoans (e.g., Haemosporida, Trypanosomatida) are transmitted by *Culicoides*. Many of these species are not lethal but do cause suffering and some can persist as chronic infections of which the impact on animal and human welfare is currently unknown. An example of a human non-viral *Culicoides*-transmitted parasite is the Neotropical *Mansonella ozzardi*. This parasite causes cavity filariasis and is transmitted by *C. furens* and *C. paraensis* in the Caribbean, Central, and South America (Buckley, 1934; Shelley and Coscarón, 2001). Both *C. furens* and *C. paraensis* are widespread in the Americas, including the U.S.; *Culicoides paraensis* extends further west than *C. furens* but the latter species can occur at higher abundance throughout its range in eastern North America. A filarial worm transmitted by *Culicoides* that can impact animal welfare is *Onchocerca cervicalis* Ralliet and Henry (Nematoda: Onchocercidae). This species infects the dermis of horses and causes severe dermatitis leading to intolerable discomfort (Mullen, 2009). Both the *C. variipennis* complex (most likely *C. sonorensis*) and *C. obsoletus* have been shown to transmit *O. cervicalis* (Foil et al., 1987). Avian blood parasites of the genera *Haemoproteus* (*Parahaemoproteus*) and *Leucocytozoon* are enzootic in North America (Linley, 1985). While these parasites are not exotic, they are ubiquitous and very little is known about the impact of infection on avian health. Some species are known to have an effect on gallinaceous bird health, such as *H. meleagridis* which is transmitted by *C.* (*Diphaomyi*a) *edeni* Wirth and Blanton, *C.* (*Ammossovia*) *aboricola* Root and Hoffman, and *C.* (*Drymodesmyia*) *hinmani* Khalaf (Linley, 1985). Infection with *Haemoproteus* spp. may have effects on fitness by reducing clutch size in

some birds and chronic infection may lead to an immunocompromised health status which may increase the severity of other parasitic infections (Knowles et al., 2010; Marzal et al., 2005).

Knowledge gaps and potential implications

Research on vector biology oftentimes lags behind research on vector-borne pathogens and their vertebrate hosts. Associated data deficiencies thus obstruct our comprehension of certain characteristics of pathogen epidemiology, such as the vectorial capacity of a population, which requires accurate parameter estimates of vector species abundance, vector competence, and feeding behaviors (Black and Moore, 2005; Purse et al., 2015). A remarkable example of large knowledge gaps about basic vector biology is demonstrated by the North American *Culicoides* species. The phylogenetic relationships among most *Culicoides* species are still largely unknown, the breadth of host choice for each species remains elusive, and the vector competence of many species has never been investigated (Harrup et al., 2015; Purse et al., 2015).

The take-home message is that we still know very little about the evolution and ecology of many *Culicoides* species and the transmission cycles of the parasites that they transmit. For example, we have highlighted the possibility that multiple *Culicoides* species may play roles in the transmission cycles of BTV and EHDV in North America. Considering that a species' ability to transmit a pathogen is driven by its genetics and ecology, and slight differences can have large effects, increased systematic research would lead to a better understanding of transmission cycles through enhanced ability to accurately identify species, estimating distribution, and quantifying intraspecific variation in ecology and genetic diversity. A current roadblock to making broad phylogenetic inferences about *Culicoides* is the fragmented nature of datasets as most molecular studies of *Culicoides* have focused on a single morphospecies group, a single morphologically identified subgenus, or species present in a narrow geographic region. These taxonomic or

regional groups, in particular the subgenera, may not be monophyletic and generating molecular phylogenies without including other subgenera could be misleading for phylogenetic inferences due to incomplete taxon sampling (Borkent and Grogan, 2009). This problem was demonstrated by the most comprehensive DNA barcoding study of European *Culicoides* subgenera and species groups to date (Ander et al., 2013). The authors found that phylogenetic patterns of previously defined species groups and subgenera were not necessarily predictable because some were either monophyletic (e.g., subgenus *Monoculicoides*), paraphyletic (e.g., subgenus *Avaritia*), or polyphyletic (e.g., subgenus *Oecacta*).

A major impediment to predicting vector-borne disease outbreaks is the presence of unknown vector species and/or cryptic species that obscure patterns of genetic variation and accurate estimates of the distributions of potential vector species (Brooks and Hoberg, 2006; Carpenter et al., 2009; Tabachnick and Black, 1995). Taxonomic accuracy is fundamental as it helps disease researchers classify and organize vector and parasite biodiversity into a framework which assists understanding and predictions of transmission dynamics. But in order for taxonomy to be useful it must be grounded on a rigorous phylogenetic foundation using the characters that are the most phylogenetically informative. In many taxa, morphological characters are excellent indicators of evolutionary history and, once identified, the character states can simplify species identification leading to rapid diagnosis. However, when the organisms of interest are extremely small, morphologically homogeneous, or we lack discriminating characters, then other characters may prove more useful. Molecular techniques such as chromosome number, DNA sequences (e.g., barcoding), DNA folding structure, polytene chromosome banding patterns, protein structure, and gene expression, to name a few, may provide significantly more power to discriminate species (Harrup et al., 2015). *Culicoides*

midges fall into the category of morphologically difficult organisms that require significant training and expertise to identify. Streamlined molecular approaches, in conjunction with other data, will greatly assist identification of vector species and delineation of all life stages (Ander et al., 2013; Besansky et al., 2003). Molecular techniques could help reduce the time and labor required for *Culicoides* species identification, if methods are properly developed, documented, and standardized. Further, sequencing of the *Culicoides sonorensis* genome, which is currently underway, will revolutionize systematics by opening the door to full genome phylogenies and the identification of gene regions that have resolution for species identification (Nayduch et al., 2014).

Research on EIDs has typically been reactive and focused on immediate problems leaving large gaps in the data about the basic biology of pathogens and vectors (Brooks and Hoberg, 2006). *Culicoides* is a genus with many species that are widespread, several with Holarctic distributions. Addressing the data deficits presented here is a daunting task which requires collaboration among laboratories with diverse expertise (Purse et al., 2015). I have undertaken a small part of the phylogenetic research needed to fill Nearctic *Culicoides* knowledge gaps (Chapter 2). But the hope is to also inspire other research groups, funding institutions, and management agencies to consider *Culicoides* as worthwhile research subjects considering the impact that BTV, EHDV, and other *Culicoides* transmitted viruses (e.g., SBV and ASHV) can have as they are constant threats to the health of North American livestock and wildlife. What is needed are multi-disciplinary partnerships that pursue a comprehensive entomological knowledge base to facilitate basic and applied science concerning arthropod-borne pathogens with the ultimate goal of improving disease management (Maclachlan and Mayo, 2013; Purse et al., 2015). Areas of expertise such as ecological modelling, behavioral ecology,

epidemiology, genetics, and veterinary diagnostics could be combined with systematics research to help us move past the current passive surveillance-based and reactive nature of *Culicoides*-borne pathogen research. Further, reliable vector species identification, in conjunction with understanding of feeding patterns through blood meal analysis could improve detection of pathogen transmission pathways. A comprehensive approach to *Culicoides* research will greatly enhance domestic animal and wildlife disease surveillance, management, and response by building a better foundation of knowledge which can be utilized to develop and implement effective mitigation strategies before an outbreak happens (Brooks and Hoberg, 2006; Daszak et al., 2004; Maclachlan and Mayo, 2013). As more emerging infectious diseases threaten human and animal welfare, research aimed at understanding the ecology and evolution of pathogen dynamics in natural systems provides fundamental information allowing development of resilient mitigation strategies that will be effective well into the future.

Table 1-*Culicoides* species in North America that have been associated with pathogen transmission. Virus abbreviations are African horse sickness virus (AHSV), Akabane virus (AKAV), Bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), West Nile virus (WNV), Oropouche virus (ORO), Schmallenberg virus (SBV), Lockern virus (LV), maindrain virus (MDV), buttonwillow virus (BWV), and vesicular stomatitis virus (VSV). Transmission of pathogens not in parentheses has been confirmed, while transmission of pathogens in parentheses is suspected based on viral isolation, PCR detection, and/or abundance and association with infected vertebrates.

Subgenus	Species	Distribution	Viruses	Non-Viral Pathogens	References
Ammossovia	*C. arboricola*	Ontario to Texas and Florida; Minnesota to Connecticut	(WNV)	*H. meleagridis*	Atkinson et al. (1988) (Sabio et al. (2006))
Avaritia	*C. boydi*	California south to Mexico	(BTV, EHDV)		Wirth and Mullens (1992)
	C. chiopterus	Europe; Alaska to Quebec; south to California and Florida	(BTV)		Carpenter et al. (2008) Dijkstra et al. (2008)
	C. obsoletus	Holarctic; south to California and Georgia	BTV-Europe (EHDV, SBV)		De Liberato et al. (2005) Mullen et al. (1985b) Veronesi et al. (2013)
	C. pusillus	Florida; south to Argentina	(BTV)		Mo et al. (1994)
Beltranmyia	*C. crepuscularis*	British Columbia to Nova Scotia; south to Costa Rica	(BTV)	Nematoda (*H. danilewski; H. fringillae; Trypanosoma* spp.)	(Becker et al. (2010); Bennett (1961); Fallis and Bennett (1961a), 1961b))
	C. sphagnumensis	Holarctic; south to West Virginia		*H. mansoni; H. velans (H. fringillae; Trypanosoma spp.)*	(Fallis and Bennett (1961a), 1961b); Khan and Fallis (1971))
Culicoides	*C. cockerellii*	British Columbia to Quebec; south to California and New Mexico	(BTV)		Kramer et al. (1990)
	C. lahontan	California, Montana, Oregon, Utah	(BTV, EHDV)		Mullens and Dada (1992b)
Diphaomyia	*C. edeni*	South Carolina to Florida; Bahamas		*H. meleagridis*	Atkinson et al. (1988)
	C. haematopotus	British Columbia to Nova Scotia; south to Honduras	(BTV)	(*H. mansoni*; Nematoda)	(Becker et al. (2010); Fallis and Bennett (1960))
Drymodesmyia	*C. hinmani*	Utah to New York; south to Texas and Florida		*H. meleagridis*	Atkinson et al. (1988)
Haematomydium	*C. debillipalpis*	Nebraska to Maryland; south to Argentina	(BTV, EHDV)		(Mullen et al. (1985a); Smith and Stallknecht (1996); Smith et al. (1996a); Smith et al. (1996b))
	C. paraensis	Colorado to Pennsylvania; south to Argentina	ORO (BTV, EHDV)	*M. ozzardi* - Neotropics	(Mullen et al. (1985b); Pinheiro et al. (1982); Pinheiro et al. (1981a); Shelley and Coscarón (2001))
Hoffmania	*C. insignis*	Alabama and Georgia to Argentina	BTV		Tanya et al. (1992)
	C. venustus	Nebraska east to Maryland; Nova Scotia and Ontario south to Louisiana and Florida	(BTV, EHDV)		Jones et al. (1983)
Monoculicoides	*C. sonorensis*	California east to South Dakota and Kansas; British Columbia and Alberta south to Mexico; Scattered east of Mississippi river	BTV, EHDV, AHSV, SBV, (AKAV, WNV, BWV, LV, MDV,VSV)	(*O. cervicalis*)	(Boorman et al. (1975); Foster et al. (1977); Foster et al. (1963); Hardy et al. (1970); Jennings and Mellor (1989); Jones (1965); Jones et al. (1977); Kramer et al. (1990); Mellor (1975); Naugle et al. (2004); Pérez de León and Tabachnick (2006); Veronesi et al., 2013)
Oecata	*C. furens*	Texas east to Atlantic Coast; Massachusetts south to Ecuador and Brazil	(BTV)	*M. ozzardi* - Neotropics	(Becker et al. (2010); Buckley (1934); Greiner et al. (1984b))
	C. stellifer	Ontario and Nova Scotia south to Mexico; All U.S. states except Washington and Oregon	(BTV, EHDV, VSV)		(Kramer et al. (1990); Mullen and Anderson (1998); Mullen et al. (1985a); Smith and Stallknecht (1996))
Selfia	*C. spp.*	Western Canada, U.S., and Mexico	(BTV, EHDV, VSV, LV, BWV, MDV)		(Kramer et al. (1990); Mullens and Dada (1992b); Walton et al., 1987)
Sylvaticulicoides	*C. biguttatus*	Nebraska east to Atlantic coast; Ontario and Nova Scotia south to Louisiana and Florida	(BTV, EHDV, WNV)		(Sabio et al., 2006; Schmidtmann et al. (1980))
Wirthomyia	*C. bottimeri*	California east to Utah; south to Arizona, New Mexico, and Texas		*H. lophortyx*	Mullens et al. (2006)
unplaced (mohave group)	*C. Mohave*	California; Arizona; Baja, Mexico	(EHDV)		Rosenstock et al. (2003)
unplaced (piliferus group)	*C. downesi*	British Columbia east to New Brunswick; south to Michigan, New York, and Maine		(*H. danilewski; H. nettionis*)	(Fallis and Bennett (1961a), 1961b); Fallis and Wood (1957))

LITERATURE CITED

Allison, A.B., Goekjian, V.H., Potgieter, A.C., Wilson, W.C., Johnson, D.J., Mertens, P.P.C., Stallknecht, D.E., 2010. Detection of a novel reassortant epizootic hemorrhagic disease virus (EHDV) in the USA containing RNA segments derived from both exotic (EHDV-6) and endemic (EHDV-2) serotypes. Journal of General Virology 91, 430-439.

Ander, M., Troell, K., Chirico, J., 2013. Barcoding of biting midges in the genus *Culicoides*: a tool for species determination. Medical and Veterinary Entomology 27, 323-331.

Anderson, C.R., Spence, L., Downs, W.G., Aitken, T.H.G., 1961. Oropouche virus: a new human disease agent from Trinidad, West Indies. The American Journal of Tropical Medicine and Hygiene 10, 574-578.

Anderson, D., Frosch, B., Outlaw, J., 2007. Economic impact of the United States cervid farming industry. Agricultural and Food Policy Center, Texas A&M University, College Station, TX.

Atchley, W.R., 1967. The *Culicoides* of New Mexico.(Diptera: Ceratopogonidae). University of Kansas Publications.

Atkinson, C.T., Forrester, D.J., Greiner, E.C., 1988. Epizootiology of *Haemoproteus meleagridis* (Protozoa: Haemosporina) in Florida: seasonal transmission and vector abundance. Journal of Medical Entomology 25, 45-51.

Becker, M.E., Reeves, W.K., Dejean, S.K., Emery, M.P., Ostlund, E.N., Foil, L.D., 2010. Detection of bluetongue virus RNA in field-collected *Culicoides* spp. (Diptera: Ceratopogonidae) following the discovery of bluetongue virus serotype 1 in white-tailed deer and cattle in Louisiana. Journal of Medical Entomology 47, 269-273.

Bennett, G.F., 1961. On the specificity and transmission of some avian trypanosomes. Canadian Journal of Zoology 39, 17-33.

Bergold, G.H., Suárez, O.M., Munz, K., 1968. Multiplication in and transmission by *Aedes aegypti* of vesicular stomatitis virus. Journal of Invertebrate Pathology 11, 406-428.

Besansky, N.J., Severson, D.W., Ferdig, M.T., 2003. DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. Trends in Parasitology 19, 545-546.

Black, W.C., Moore, C.G., 2005. Population biology as a tool to study vector-borne diseases. In: Marquardt, W.C. (Ed.), Biology of Disease Vectors. Elsevier Academic Press, Burlington, MA, USA, pp. 187-206.

Blanton, F.S., Wirth, W.W., 1979. The sand flies (*Culicoides*) of Florida (Diptera: Ceratopogonidae). Florida Department of Agriculture and Consumer Services, Gainesville, Florida, USA.

Boorman, J., Mellor, P.S., Penn, M., Jennings, M., 1975. The growth of African horse-sickness virus in embryonated hen eggs and the transmission of virus by *Culicoides variipennis* Coquillett (Diptera, Ceratopogonidae). Archives of Virology 47, 343-349.

Borkent, A., 2005. The biting midges, the Ceratopogonidae (Diptera). In: Marquardt, W.C. (Ed.), Biology of Disease Vectors. Elsevier Academic Press, London, UK, pp. 113-126.

Borkent, A., 2015. World species of biting midges (Diptera: Ceratopogonidae). Royal British Columbia Museum, American Museum of Natural History, and Instituto Nacional de Biodiversidad, Salmon Arm, British Columbia, Canada.

Borkent, A., Grogan, W.L., 2009. Catalog of the New World biting midges north of Mexico (Diptera: Ceratopogonidae). Zootaxa 2273, 1-48.

Bram, R.A., George, J.E., Reichard, R.E., Tabachnick, W.J., 2002. Threat of foreign arthropod-borne pathogens to livestock in the United States. Journal of Medical Entomology 39, 405-416.

Brooks, D.R., Hoberg, E.P., 2006. Systematics and emerging infectious diseases: From management to solution. Journal of Parasitology 92, 426-429.

Buckley, J.J.C., 1934. On the development, in *Culicoides furens* Poey, of *Filaria* (= *Mansonella*) *ozzardi* Manson, 1897. Journal of Helminthology 12, 99-118.

Campbell, C.L., Vandyke, K.A., Letchworth, G.J., Drolet, B.S., Hanekamp, T., Wilson, W.C., 2005. Midgut and salivary gland transcriptomes of the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae). Insect Molecular Biology 14, 121-136.

Caracappa, S., Torina, A., Guercio, A., Vitale, F., Calabro, A., Purpari, G., Ferrantelli, V., Vitale, M., Mellor, P., 2003. Identification of a novel bluetongue virus vector species of *Culicoides* in Sicily. Veterinary Record 153, 71-74.

Carpenter, S., Groschup, M.H., Garros, C., Felippe-Bauer, M.L., Purse, B.V., 2013. *Culicoides* biting midges, arboviruses and public health in Europe. Antiviral Research 100, 102-113.

Carpenter, S., Szmaragd, C., Barber, J., Labuschagne, K., Gubbins, S., Mellor, P., 2008. An assessment of *Culicoides* surveillance techniques in northern Europe: have we underestimated a potential bluetongue virus vector? Journal of Applied Ecology 45, 1237-1245.

Carpenter, S., Wilson, A., Mellor, P.S., 2009. *Culicoides* and the emergence of bluetongue virus in northern Europe. Trends in Microbiology 17, 172-178.

Darpel, K.E., Batten, C.A., Veronesi, E., Shaw, A.E., Anthony, S., Bachanek-Bankowska, K., Kgosana, L., bin-Tarif, A., Carpenter, S., Muller-Doblies, U.U., Takamatsu, H.H.,

Mellor, P.S., Mertens, P.P., Oura, C.A., 2007. Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. Vet Rec 161, 253-261.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287, 443-449.

Daszak, P., Tabor, G.M., Kilpatrick, A.M., Epstein, J.O.N., Plowright, R., 2004. Conservation medicine and a new agenda for emerging diseases. Annals of the New York Academy of Sciences 1026, 1-11.

De Liberato, C., Scavia, G., Lorenzetti, R., Scaramozzino, P., Amaddeo, D., Cardeti, G., Scicluna, M., Ferrari, G., Autorino, G.L., 2005. Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. The Veterinary Record 156, 301-304.

Dijkstra, E., van der Ven, I.J.K., Meiswinkel, R., Hölzel, D.R., van Rijn, P.A., Meiswinkel, R., 2008. *Culicoides chiopterus* as a potential vector of bluetongue virus in Europe. Veterinary Record 162, 422.

Downes, J.A., 1978. The *Culicoides variipennis* complex: a necessary re-alignment of nomenclature (Diptera: Ceratopogonidae). The Canadian Entomologist 110, 63-69.

Drolet, B.S., Campbell, C.L., Stuart, M.A., Wilson, W.C., 2005. Vector competence of *Culicoides sonorensis* (Diptera: Ceratopogonidae) for vesicular stomatitis virus. Journal of Medical Entomology 42, 409-418.

Enserink, M., 2008. Exotic disease of farm animals tests Europe's responses. Science 319, 710-711.

Ewald, P.W., 1995. The evolution of virulence: a unifying link between parasitology and ecology. Journal of Parasitology 81, 659-669.

Falconi, C., López-Olvera, J.R., Gortázar, C., 2011. BTV infection in wild ruminants, with emphasis on red deer: A review. Veterinary Microbiology 151, 209-219.

Fallis, A.M., Bennett, G.F., 1960. Description of *Haemoproteus canachites* N. sp. (Sporozoa:Haemopoteidae) and sporogony in *Culicoides* (Diptera:Ceratopogonidae). Canadian Journal of Zoology 38, 455-464.

Fallis, A.M., Bennett, G.F., 1961a. Ceratopogonidae as intermediate hosts for *Haemoproteus* and other parasites. Mosquito News 21, 21-28.

Fallis, A.M., Bennett, G.F., 1961b. Sporogony of *Leucocytozoon* and *Haemoproteus* in sumuliids and ceratopogonids and a reclassification of the Haemosporidiida. Canadian Journal of Zoology 39, 215-228.

Fallis, A.M., Wood, D.M., 1957. Biting midges (Diptera: Ceratopogonidae) as intermediate hosts for *Haemoproteus* of ducks Canadian Journal of Zoology 35, 425-435.

Foil, L.D., Klei, T.R., Miller, R.I., Church, G.E., Foil, C.S., French, D.D., Smith, J.N., 1987. Seasonal changes in density and tissue distribution of *Onchocerca cervicalis* Microfilariae in ponies and related changes in *Culicoides variipennis* populations in Louisiana. The Journal of Parasitology 73, 320-326.

Foster, N., Breckon, R., Luedke, A., Jones, R., 1977. Transmission of two strains of epizootic hemorrhagic disease virus in deer by *Culicoides variipennis*. Journal of Wildlife Diseases 13, 9-16.

Foster, N., Jones, R., McCrory, B., 1963. Preliminary investigations on insect transmission of bluetongue virus in sheep. American Journal of Veterinary Research 24, 1195.

García-Bocanegra, I., Arenas-Montes, A., Lorca-Oró, C., Pujols, J., González, M.A., Napp, S., Gómez-Guillamón, F., Zorrilla, I., Miguel, E.S., Arenas, A., 2011. Role of wild ruminants in the epidemiology of bluetongue virus serotypes 1, 4 and 8 in Spain. Veterinary Research 42, 88.

Gaydos, J.K., Crum, J.M., Davidson, W.R., Cross, S.S., Owen, S.F., Stallknecht, D.E., 2004. Epizootiology of an epizootic hemorrhagic disease outbreak in West Virginia. Journal of Wildlife Diseases 40, 383-393.

Gibbs, E.P.J., Tabachnick, W.J., Holt, T.J., Stallknecht, D.E., 2008. U.S. concerns over bluetongue. Science 320, 872.

Gould, A.R., Hyatt, A.D., 1994. The orbivirus genus. Diversity, structure, replication and phylogenetic relationships. Comparative Immunology, Microbiology and Infectious Diseases 17, 163-188.

Grado, S.C., Hunt, K.M., Whiteside, M.W., 2007. Economic impacts of white-tailed deer hunting in Mississippi. Proceedings of the Southeastern Association of Fish and Wildlife Agencies 61, 59-67.

Greiner, E., Barber, T., Pearson, J., Kramer, W., Gibbs, E., 1984a. Orbiviruses from *Culicoides* in Florida. Progress in Clinical and Biological Research 178, 195-200.

Greiner, E.C., Garris, G.I., Rollo, R.T., Knausenberger, W.I., Jones, J.E., Gibbs, E.P.J., 1984b. Preliminary studies on the *Culicoides* spp. as potential vectors of bluetongue in the Caribbean region. Preventive Veterinary Medicine 2, 389-399.

Grogan Jr., W.L., Phillips, R.A., 2008. A new species of biting midge in the subgenus *Monoculicoides* of *Culicoides* from Utah (Diptera: Ceratopogonidae). Proceedings of the Entomological Society of Washington 110, 196-203.

Harbach, R.E., 2004. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bull Entomol Res 94, 537-553.

Hardy, J.L., Scrivani, R.P., Lyness, R.N., Nelson, R.L., Boberts, D.R., 1970. Ecologic studies on buttonwillow virus in Kern County, California, 1961–1968. The American Journal of Tropical Medicine and Hygiene 19, 552-563.

Harrup, L.E., Bellis, G.A., Balenghien, T., Garros, C., 2015. *Culicoides* Latreille (Diptera: Ceratopogonidae) taxonomy: Current challenges and future directions. Infection, Genetics and Evolution 30, 249-266.

Harrus, S., Baneth, G., 2005. Drivers for the emergence and re-emergence of vector-borne protozoal and bacterial diseases. International Journal of for Parasitology 35, 1309-1318.

Hensleigh, D.A., Atchley, W.R., 1977. Morphometric variability in natural and laboratory populations of *Culicoides variipennis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 14, 379-386.

Hoar, B.R., Carpenter, T.E., Singer, R.S., Gardner, I.A., 2003. Regional risk of exporting cattle seropositive for bluetongue virus from the United States. American Journal of Veterinary Research 64, 520-529.

Hoffmann, B., Bauer, B., Bauer, C., Bätza, H.-J., Beer, M., Clausen, P.-H., Geier, M., Gethmann, J.M., Kiel, E., Liebisch, G., Liebisch, A., Mehlhorn, H., Schaub, G.A., Werner, D., Conraths, F.J., 2009. Monitoring of putative vectors of bluetongue virus serotype 8, Germany. Emerging Infectious Diseases 15, 1481-1484.

Holbrook, F.R., Tabachnick, W.J., 1995. *Culicoides variipennis* (Diptera: Ceratopogonidae) complex in California. Journal of Medical Entomology 32, 413-419.

Holbrook, F.R., Tabachnick, W.J., Brady, R., 1996. Genetic variation in populations of *Culicoides variipennis* complex in the six New England states, U.S.A. Medical and Veterinary Entomology 10, 173-180.

Holbrook, F.R., Tabachnick, W.J., Schmidtmann, E.T., McKinnon, C.N., Bobian, R.J., Grogan Jr, W.L., 2000. Sympatry in the *Culicoides variipennis* complex (Diptera: Ceratopogonidae): a taxonomic reassessment. Journal of Medical Entomology 37, 65-76.

Hoogendam, K., 2007. International study on the economic consequences of outbreaks of bluetongue serotype 8 in north-western Europe. Van Hall Institute, Leeuwarden, The Netherlands.

Hourrigan, J.L., Klingsporn, A.L., 1975. Epizootiology of bluetongue: the situation in the United States of America. Australian Veterinary Journal 51, 203-208.

Huerta, H., Rodríguez Castrejón, A.M., Grogan Jr, W.L., Ibánez-Bernal, S., 2012. New records of biting midges of the genus *Culicoides* Latreille from Mexico (Diptera: Ceratopogonidae). Insecta Mundi 0211, 1-20.

Jackson, J.A., Tinsley, R.C., 2005. Geographic and within-population structure in variable resistance to parasite species and strains in a vertebrate host. Int J Parasitol 35, 29-37.

Jamnback, H., Wirth, W., 1963. The species of *Culicoides* related to *obsoletus* in eastern North America (Diptera: Ceratopogonidae). Annals of the Entomological Society of America 56, 185-198.

Jennings, M., Mellor, P.S., 1989. *Culicoides*: Biological vectors of akabane virus. Veterinary Microbiology 21, 125-131.

Johnson, D.J., Ostlund, E.N., Stallknecht, D.E., Goekjian, V.H., Jenkins-Moore, M., Harris, S.C., 2006. First report of bluetongue virus serotype 1 isolated from a white-tailed deer in the United States. Journal of Veterinary Diagnostic Investigation 18, 398-401.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature 451, 990-993.

Jones, R., 1965. Epidemiological notes: incidence of *Culicoides variipennis* in an outbreak of bluetongue disease. Mosquito News 25, 217-218.

Jones, R., Schmidtmann, E., Foster, N., 1983. Vector-competence studies for bluetongue and epizootic hemorrhagic disease viruses with *Culicoides venustus* (Ceratopogonidae). Mosquito News 43.

Jones, R.H., Roughton, R.D., Foster, N.M., Bando, B.M., 1977. *Culicoides*, the vector of epizootic hemorrhagic disease deer in Kentucky in 1971. Journal of Wildlife Diseases 13, 2-8.

Kedmi, M., Van Straten, M., Ezra, E., Galon, N., Klement, E., 2010. Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds. Journal of Dairy Science 93, 2486-2495.

Khalaf, K., 1954. The speciation of the genus *Culicoides* (Diptera, Heleidae). Annals of the Entomological Society of America 47, 34-51.

Khan, R.A., Fallis, A.M., 1971. A note on the sporogony of *Parahaemoproteus velans* (= *Haemoproteus velans* Coatney and Roudabush) (Haemosporidia: Haemoproteidae) in species of *Culicoides*. Canadian Journal of Zoology 49, 420-421.

Kilpatrick, A.M., 2011. Globalization, land use, and the invasion of West Nile virus. Science 334, 323-327.

Knowles, S.C.L., Palinauskas, V., Sheldon, B.C., 2010. Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. Journal of Evolutionary Biology 23, 557-569.

Koenraadt, C., Balenghien, T., Carpenter, S., Ducheyne, E., Elbers, A., Fife, M., Garros, C., Ibanez-Justicia, A., Kampen, H., Kormelink, R., Losson, B., van der Poel, W., De Regge, N., van Rijn, P., Sanders, C., Schaffner, F., Sloet van Oldruitenborgh-Oosterbaan, M., Takken, W., Werner, D., Seelig, F., 2014. Bluetongue, Schmallenberg - what is next? *Culicoides*-borne viral diseases in the 21st Century. BMC Veterinary Research 10, 77.

Kramer, W.L., Greiner, E.C., Gibbs, E.P.J., 1985a. Seasonal variations in populations size, fecundity, and parity rates of *Culicoides insignis* (Diptera: Ceratopogonidae) in Florida, USA. Journal of Medical Entomology 22, 163-169.

Kramer, W.L., Greiner, E.C., Gibbs, E.P.J., 1985b. A survey of *Culicoides* midges (Diptera: Ceratopogonidae) associated with cattle operations in Florida, USA. Journal of Medical Entomology 22, 153-162.

Kramer, W.L., Jones, R.H., Holbrook, F.R., Walton, T.E., Calisher, C.H., 1990. Isolation of arboviruses from *Culicoides* midges (Diptera: Ceratopogonidae) in Colorado during an epizootic of vesicular stomatitis New Jersey. Journal of Medical Entomology 27, 487-493.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile

virus responsible for an outbreak of encephalitis in the Northeastern United States. Science 286, 2333-2337.

Leather, S.R., 2009. Taxonomic chauvinism threatens the future of entomology. Biologist 56, 10.

Linden, A., Desmecht, D., Volpe, R., Wirtgen, M., Gregoire, F., Pirson, J., Paternostre, J., Kleijnen, D., Schirrmeier, H., Beer, M., Garigliany, M.-M., 2012. Epizootic spread of Schmallenberg virus among wild cervids, Belgium, Fall 2011. Emerging Infectious Diseases 18, 2006-2008.

Linley, J.R., 1985. Biting midges (Diptera: Ceratopogonidae) as vectors of nonviral animal pathogens. Journal of Medical Entomology 22, 589-599.

Maclachlan, N., Drew, C., Darpel, K., Worwa, G., 2009. The pathology and pathogenesis of bluetongue. Journal of comparative pathology 141, 1-16.

MacLachlan, N.J., Guthrie, A.J., 2010. Re-emergence of bluetongue, African horse sickness, and other *Orbivirus* diseases. Vet. Res. 41, 35.

Maclachlan, N.J., Mayo, C.E., 2013. Potential strategies for control of bluetongue, a globally emerging, *Culicoides*-transmitted viral disease of ruminant livestock and wildlife. Antiviral Research 99, 79-90.

Marzal, A., Lope, F., Navarro, C., Møller, A., 2005. Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia 142, 541-545.

Mead, D.G., Ramberg, F.B., Besselsen, D.G., Maré, C.J., 2000. Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice. Science 287, 485-487.

Mecham, J.O., Stallknecht, D., Wilson, W.C., 2003. The S7 gene and VP7 protein are highly conserved among temporally and geographically distinct American isolates of epizootic hemorrhagic disease virus. Virus research 94, 129-133.

Meinswinkel, R., Nevill, E.M., Venter, G.J., 1994. Vectors: *Culicoides* spp. In: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. (Eds.), Infectious disease of livestock with special reference to Southern Africa. Oxford University Press, Cape Town, pp. 68-89.

Meiswinkel, R., Van Rijn, P., Leijs, P., Goffredo, M., 2007. Potential new *Culicoides* vector of bluetongue virus in northern Europe. Veterinary Record 161, 564-565.

Mellor, P.S., 1975. Studies on *Onchocerca cervicalis* Railliet and Henry 1910: V. The development of *Onchocerca cervicalis* larvae in the vectors. Journal of Helminthology 49, 33-42.

Mellor, P.S., Boorman, J., Baylis, M., 2000. *Culicoides* biting midges: their role as arbovirus vectors. Annual Review of Entomology 45, 307-340.

Mellor, P.S., Wittmann, E.J., 2002. Bluetongue virus in the Mediterranean Basin 1998-2001. Vet J 164, 20-37.

Miller, M.M., Brown, J., Cornish, T., Johnson, G., Mecham, J.O., Reeves, W.K., Wilson, W., 2010. Investigation of a bluetongue disease epizootic caused by bluetongue virus serotype 17 in sheep in Wyoming. Journal of the American Veterinary Medical Association 237, 955-959.

Mo, C.L., Thompson, L.H., Homan, E.J., Oviedo, M.T., Greiner, E.C., González, J., Sáenz, M.R., 1994. Bluetongue virus isolations from vectors and ruminants in Central America and the Caribbean. American Journal of Veterinary Research 55, 211-215.

Montana Fish, W., and Parks, 2011. Press release: EHD takes toll on Eastern Montana white-tailed deer.

Mullen, G., Anderson, R., 1998. Transmission of orbiviruses by *Culicoides* Latreille species (Ceratopogonidae) among cattle and white-tailed deer in the southeastern United States. Abstracts Volume. Fourth International Congress of Dipterology, pp. 155-156.

Mullen, G., Jones, R., Braverman, Y., Nusbaum, K., 1985a. Laboratory infections of *Culicoides debilipalpis* and *C. stellifer* (Diptera: Ceratopogonidae) with bluetongue virus. Progress in clinical and biological research 178, 239.

Mullen, G.R., 2009. Biting Midges (Ceratopogonidae). In: Mullen, G.R., Durden, L.A. (Eds.), Medical and Veterinary Entomology. Academic Press, Burlington, MA, pp. XX-XX.

Mullen, G.R., Hayes, M.E., Nusbaum, K.E., 1985b. Potential vectors of bluetongue and epizootic hemorrhagic disease viruses of cattle and white-tailed deer in Alabama. Progress in Clinical and Biological Research 178, 201-206.

Mullens, B.A., Cardona, C.J., McClellan, L., Szijj, C.E., Owen, J.P., 2006. *Culicoides bottimeri* as a vector of *Haemoproteus lophortyx* to quail in California, USA. Veterinary Parasitology 140, 35-43.

Mullens, B.A., Dada, C.E., 1992a. Insects feeding on desert bighorn sheep, domestic rabbits, and japanese quail in the Santa Rosa Mountains of Southern California. Journal of Wildlife Diseases 28, 476-480.

Mullens, B.A., Dada, C.E., 1992b. Spatial and seasonal distribution of potential vectors of hemorrhagic disease viruses to peninsular bighorn sheep in the Santa Rosa mountains of southern California. Journal of Wildlife Diseases 28, 192-205.

Naugle, D.E., Aldridge, C.L., Walker, B.L., Cornish, T.E., Moynahan, B.J., Holloran, M.J., Brown, K., Johnson, G.D., Schmidtmann, E.T., Mayer, R.T., Kato, C.Y., Matchett, M.R., Christiansen, T.J., Cook, W.E., Creekmore, T., Falise, R.D., Thomas Rinkes, E., Boyce, M.S., 2004. West Nile virus: pending crisis for greater sage-grouse. Ecology Letters 7, 704-713.

Nayduch, D., Cohnstaedt, L.W., Saski, C., Lawson, D., Kersey, P., Fife, M., Carpenter, S., 2014. Studying *Culicoides* vectors of BTV in the post-genomic era: Resources, bottlenecks to progress and future directions. Virus Research 182, 43-49.

Nettles, V., Davidson, W., Stallknecht, D., 1992. Surveillance for hemorrhagic disease in white-tailed deer and other wild ruminants, 1980–1989. Proceedings of the annual conference of the Southeastern Association of Fish and Wildlife Agencies, pp. 138-146.

Noon, T.H., Wesche, S.L., Cagle, D., Mead, D.G., Bicknell, E.J., Bradley, G.A., Riplog-Peterson, S., Edsall, D., Reggiardo, C., 2002. Hemorrhagic disease in bighorn sheep in Arizona. Journal of wildlife diseases 38, 172-176.

Osborne, C.J., Mayo, C.E., Mullens, B.A., McDermott, E.G., Gerry, A.C., Reisen, W.K., MacLachlan, N.J., 2015. Lack of evidence for laboratory and natural vertical transmission of bluetongue virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae).

Ostlund, E., Moser, K., Johnson, D., Pearson, J., Schmitt, B., 2004. Distribution of bluetongue in the United States of America, 1991–2002. Vet Ital 40, 83-88.

Packer, L., Grixti, J.C., Roughley, R.E., Hanner, R., 2009. The status of taxonomy in Canada and the impact of DNA barcoding. Canadian Journal of Zoology 87, 1097-1110.

Pagès, N., Muñoz-Muñoz, F., Talavera, S., Sarto, V., Lorca, C., Núñez, J.I., 2009. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides*

and development of species-specific PCR assays based on barcode regions. Veterinary Parasitology 165, 298-310.

Pérez de León, A.A., O'Toole, D., Tabachnick, W.J., 2006. Infection of Guinea pigs with vesicular stomatitis New Jersey virus transmitted by *Culicoides sonorensis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 43, 568-573.

Pérez de León, A.A., Tabachnick, W.J., 2006. Transmission of vesicular stomatitis New Jersey virus to cattle by the biting midge *Culicoides sonorensis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 43, 323-329.

Petersen, L.R., Hayes, E.B., 2008. West Nile virus in the Americas. Medical Clinics of North America 92, 1307-1322.

Pinheiro, F., Travassos da Rosa, A., Gomes, M., LeDuc, J., Hoch, A., 1982. Transmission of Oropouche virus from man to hamster by the midge *Culicoides paraensis*. Science 215, 1251-1253.

Pinheiro, F.P., Hoch, A.L., Gomes, M.L., Roberts, D.R., 1981a. Oropouche virus. IV. Laboratory transmission by *Culicoides paraensis*. The American Journal of Tropical Medicine and Hygiene 30, 172-176.

Pinheiro, F.P., Travassos da Rosa, A., Travassos da Rosa, J., Ishak, R., GOMES, M., OUVA, O., LeDuc, J., 1981b. Oropouche Virus: A review of clinical, epidemiological and ecological findings. American Journal of Tropical Medicine and Hygiene 30, 149-160.

ProMED-mail, 2012. Epizootic hemorrhagic disease, cervids - USA (23): (NE)

Purse, B.V., Carpenter, S., Venter, G.J., Bellis, G., Mullens, B.A., 2015. Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of *Culicoides*-borne viruses. Annual Review of Entomology 60, 373-392.

Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P.C., Baylis, M., 2005. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Micro 3, 171-181.

Rosenstock, S., Ramberg, F., Collins, J., Rabe, M., 2003. *Culicoides mohave* (Diptera: Ceratopogonidae): New occurrence records and potential role in transmission of hemorrhagic disease. Journal of Medical Entomology 40, 577-579.

Ruiz-Fons, F., Sánchez-Matamoros, A., Gortázar, C., Sánchez-Vizcaíno, J.M., 2014. The role of wildlife in bluetongue virus maintenance in Europe: Lessons learned after the natural infection in Spain. Virus Research 182, 50-58.

Sabio, I.J., Mackay, A.J., Roy, A., Foil, L.D., 2006. Detection of West Nile virus RNA in pools of three species of Ceratopogonids (Diptera: Ceratopogonidae) collected in Louisiana. Journal of Medical Entomology 43, 1020-1022.

Samal, S.K., El-Hussein, A., Holbrook, F.R., Beaty, B.J., Ramig, R.F., 1987. Mixed infection of *Culicoides variipennis* with bluetongue virus serotypes 10 and 17: evidence for high frequency reassortment in the vector. Journal of General Virology 68, 2319-2329.

Savini, G., Afonso, A., Mellor, P., Aradaib, I., Yadin, H., Sanaa, M., Wilson, W., Monaco, F., Domingo, M., 2011. Epizootic heamorragic disease. Research in veterinary science 91, 1-17.

Schmidtmann, E.T., Herrero, M.V., Green, A.L., Dargatz, D.A., Rodriquez, J.M., Walton, T.E., 2011. Distribution of *Culicoides sonorensis* (Diptera: Ceratopogonidae) in Nebraska, South Dakota, and North Dakota: clarifying the epidemiology of bluetongue disease in the northern Great Plains region of the United States. Journal of Medical Entomology 48, 634-643.

Schmidtmann, E.T., Jones, C.J., Gollands, B., 1980. Comparative host-seeking activity of *Culicoides* (Diptera: Ceratopogonidae) attracted to pastured livestock in central New York state, USA. Journal of Medical Entomology 17, 221-231.

Schwenkenbecher, J., Mordue, A., Piertney, S., 2009. Phylogenetic analysis indicates that *Culicoides dewulfi* should not be considered part of the *Culicoides obsoletus* complex. Bulletin of entomological research 99, 371.

Sellers, R.F., Maarouf, A.R., 1989. Trajectory analysis and bluetongue virus serotype 2 in Florida 1982. Canadian Journal of Veterinary Research 53, 100-102.

Shelley, A., Coscarón, S., 2001. Simuliid blackflies (Diptera: Simuliidae) and ceratopogonid midges (Diptera: Ceratopogonidae) as vectors of *Mansonella ozzardi* (Nematoda: Onchocercidae) in northern Argentina. Memórias do Instituto Oswaldo Cruz 96, 451-458.

Smith, K.E., Stallknecht, D.E., 1996. *Culicoides* (Diptera: Ceratopogonidae) collected during epizootics of hemorrhagic disease among captive white-tailed deer. Journal of Medical Entomology 33, 507-510.

Smith, K.E., Stallknecht, D.E., Nettles, V.F., 1996a. Experimental infection of *Culicoides lahillei* (Diptera: Ceratopogonidae) with epizootic hemorrhagic disease virus serotype 2 (Orbivirus: Reoviridae). Journal of Medical Entomology 33, 117-122.

Smith, K.E., Stallknecht, D.E., Sewell, C.T., Rollor, E.A., Mullen, G.R., Anderson, R.R., 1996b. Monitoring of *Culicoides* spp. at a site enzootic for hemorrhagic disease in white-tailed deer in Georgia, USA. Journal of Wildlife Diseases 32, 627-642.

Spinelli, G.R., Ronderos, M.M., 1997. Description of the male of *Culicoides lahillei* and revalidation of *Culicoides debilipalpis* (Diptera: Ceratopogonidae). Transactions of the American Entomological Society 123, 303-309.

Stallknecht, D., Howerth, E., 2004. Epidemiology of bluetongue and epizootic haemorrhagic disease in wildlife: surveillance methods. Vet Ital 40, 203-207.

Sutherst, R.W., 2004. Global change and human vulnerability to vector-borne diseases. Clinical microbiology reviews 17, 136-173.

Swanson, D., 2012. Ecology and phylogeny of the biting-midge genus *Culicoides* (Diptera: Ceratopogonidae). Entomology, Soils, and Plant Sciences. Clemson University, Clemson, SC, USA.

Tabachnick, W.J., 1990. Genetic variation in laboratory and field populations of the vector of bluetongue virus, *Culicoides variipennis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 27, 24-30.

Tabachnick, W.J., 1992. Genetic differentiation among populations of *Culicoides variipennis* (Diptera: Ceratopogonidae), the North American Vector of bluetongue virus. Annals of the Entomological Society of America 85, 140-147.

Tabachnick, W.J., 1996. *Culicoides variipennis* and bluetongue-virus epidemiology in the United States. Annual Review of Entomology 41, 23-43.

Tabachnick, W.J., 2004. *Culicoides* and the global epidemiology of bluetongue virus. Veterinaria Italiana 40, 145-150.

Tabachnick, W.J., 2010. Challenges in predicting climate and environmental effects on vector-borne disease episystems in a changing world. Journal of Experimental Biology 213, 946-954.

Tabachnick, W.J., Black, W.C., 1995. Making a case for molecular population genetic studies of arthropod vectors. Parasitology Today 11, 27-30.

Tabachnick, W.J., Holbrook, F.R., 1992. The *Culicoides variipennis* complex and the distribution of bluetongue viruses in the United States. Proceedings of the United States Animal Health Association 96, 207-212.

Tanya, V.N., Greiner, E.C., Gibbs, E.P.J., 1992. Evaluation of *Culicoides insignis* (Diptera: Ceratopogonidae) as a vector of bluetongue virus. Veterinary Microbiology 32, 1-14.

Tarlinton, R., Daly, J., Dunham, S., Kydd, J., 2012. The challenge of Schmallenberg virus emergence in Europe. The Veterinary Journal 194, 10-18.

Tosh, R.B., Chaniotis, B.N., Johnson, K.M., 1971. Vesicular stomatitus Indiana serotype: mulitiplication in and transmission by experimentally infected phlebotomine sandflies (*Lutzomyia trapidoi*) American Journal of Epidemiology 93, 491-495.

Valkiunas, G., 2004. Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton, Florida.

Veronesi, E., Henstock, M., Gubbins, S., Batten, C., Manley, R., Barber, J., Hoffmann, B., Beer, M., Attoui, H., Mertens, P.P.C., Carpenter, S., 2013. Implicating *Culicoides* biting bidges as vectors of Schmallenberg virus using semi-quantitative rt-PCR. PLoS ONE 8, e57747.

Vigil, S.L., Wlodkowski, J.C., Parris, J., Edwards de Vargas, S., Shaw, D., Cleveland, C., Grogan Jr, W.L., Corn, J.L., 2014. New records of biting midges of the genus *Culicoides* Latreille from the southeastern United States (Diptera: Ceratopogonidae).

Walton, T.E., Webb, P.A., Kramer, W.L., Smith, G.C., Davis, T., Holbrook, F.R., Moore, C.G., Schiefer, T.J., Jones, R.H., Janney, G.C., 1987. Epizootic vesicular stomatitis in Colorado, 1982: epidemiologic and entomologic Studies. The American Journal of Tropical Medicine and Hygiene 36, 166-176.

Weaver, S.C., Barrett, A.D., 2004. Transmission cycles, host range, evolution and emergence of arboviral disease. Nature reviews. Microbiology 2, 789-801.

Wenk, C.E., Kaufmann, C., Schaffner, F., Mathis, A., 2012. Molecular characterization of Swiss Ceratopogonidae (Diptera) and evaluation of real-time PCR assays for the identification of *Culicoides* biting midges. Veterinary Parasitology 184, 258-266.

Wheeler, Q.D., Raven, P.H., Wilson, E.O., 2004. Taxonomy: impediment or expedient? Science 303, 285.

White, D.M., Wilson, W.C., Blair, C.D., Beaty, B.J., 2005. Studies on overwintering of bluetongue viruses in insects. Journal of General Virology 86, 453-462.

Wiesers-Chimpf, L., Wilson, W., French, D., Bahama, A., Foil, L., 1993. Bluetongue virus in sheep and cattle and *Culicoides variipennis* and *C. stellifer* (Diptera: Ceratopogonidae) in Louisiana. Journal of medical entomology 30, 719-724.

Wilson, A., Darpel, K., Mellor, P.S., 2008. Where does bluetongue virus sleep in the winter? PLoS biology 6, e210.

Wilson, A.J., Mellor, P.S., 2009. Bluetongue in Europe: past, present and future. Philosophical Transactions of the Royal Society B: Biological Sciences 364, 2669-2681.

Wilson, W.C., Mecham, J.O., Schmidtmann, E.T., Sanchez, C.J., Herrero, M., Lager, I., 2009. Current status of bluetongue in the Americas. In: Mellor, P.S., Baylis, M., Mertens, P.P.C. (Eds.), Bluetongue. Academic Press, London, UK, pp. 197-222.

Wirth, W., Morris, C., 1985. The taxonomic complex, *Culicoides variipennis*. In: Barber, T., Jochim, M. (Eds.), Bluetongue and related orbiviruses, Liss, New York, pp. 165-175.

Wirth, W.W., Jones, R.H., 1957. The North American subspecies of *Culicoides variipennis* (Diptera, Heleidae). United States Deptartment of Agriculture, Washington, D.C., U.S.A.

Wirth, W.W., Mullens, B.A., 1992. *Culicoides boydi* (Diptera: Ceratopogonidae): A potential vector of hemorrhagic disease viruses to desert bighorn sheep in Southern California. Journal of Medical Entomology 29, 1006-1010.

Work, T.M., Jessup, D.A., Sawyer, M.M., 1992. Experimental bluetongue and epizootic hemorrhagic disease virus infection in California black-tailed deer. Journal of Wildlife Diseases 28, 623-628.

CHAPTER 2

Multi-locus phylogeny reveals extensive taxonomic uncertainty in the *Culicoides variipennis* species complex (Diptera: Ceratopogonidae)

Introduction

The field of systematics can greatly enhance the study of emerging infectious diseases (EIDs) and bolster disease management programs by providing pertinent information about the evolution and ecology of pathogens and/or vectors. Systematics provides framework for discovery of new pathogen and vector lineages, and leads to increased accuracy in discriminating species (e.g., Pagès et al., 2009). Complex biological processes such as co-evolution, ecological specialization, and biogeographic events that shaped the distribution and diversification of pathogens and parasites can be inferred using systematics, particularly molecular phylogenetics (Hellgren et al., 2009; Kitchen et al., 2011; Martinsen et al., 2008; Waltari et al., 2007). Molecular phylogenetic studies can be used to test current taxonomic hypotheses of both vectors and pathogens. Further hypothesis testing refines taxonomic designations and places them in the context of the underlying phylogenetic relationships and historical biogeography. The application of these refined taxonomies will be rigorous species identification that enables EID management to be proactive rather than reactive (Brooks and Hoberg, 2006; Harbach, 2004).

Emerging infectious diseases at the wildlife-livestock interface have drawn interest researchers in recent decades. The main driver behind this increased attention has been the zoonotic nature of many of these pathogens and their impacts on agricultural production and, in some cases, human health (Jenkins et al., 2015; Wiethoelter et al., 2015). Due to this heightened

interest, increases in disease incidence in wildlife, which are linked to human activities, have led to many concerns about the disruption of ecosystem processes from disease outbreaks (Tompkins et al., 2015). One group of pathogens that are highly virulent and that have had large negative effects on animal and ecosystem health are those transmitted by arthropod vectors (Ewald, 1995; Jones et al., 2008). Vector-pathogen-host transmission networks are complex, and, despite the increased appreciation for their importance, there is a lack of fundamental biological knowledge about these systems, in particular vector taxonomy and phylogeny (Harrup et al., 2015; Tabachnick, 2004; Tabachnick and Black, 1995). Many vector species are small in size, fragile, have limited diagnostic morphological characters, and many often reflect cryptic species, which further complicates species identification. The inherent difficulties of studying arthropod vectors have severely impeded research and surveillance of vector-borne pathogens of non-human animals (Carpenter et al., 2009; Harrup et al., 2015).

Biting midges in the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) are vectors of some of the most devastating animal pathogens (Mellor et al., 2000). *Culicoides* are small blood-feeding flies (2-3 mm) that are highly abundant and globally ubiquitous (except New Zealand and Antarctica). Over 1400 species are currently described with only a small percentage considered competent pathogen vectors (Borkent, 2015). Among these vectors are species that transmit multiple viruses that have high morbidity and mortality in livestock and wildlife populations worldwide. The main viruses of concern to livestock and wildlife that are transmitted by *Culicoides* are bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), African horse sickness virus (AHSV), and Schmallenberg virus (SBV) (Du Toit, 1944; Foster et al., 1977; MacLachlan and Guthrie, 2010; Tarlinton et al., 2012).

The limitations of *Culicoides*-borne disease management resulting from inadequate scientific data, in particular incomplete taxonomy and phylogeny, were revealed during the European outbreaks of BTV serotype 8 (BTV-8) in the late 1990's and early 2000's. The transmission of BTV in northern Europe was not thought possible because the native *Culicoides* species were not considered competent vectors (Carpenter et al., 2009). However, the BTV-8 outbreak quickly moved across the continent, reaching Scandinavian countries. Subsequent research revealed that multiple native *Culicoides* species were likely complexes of cryptic species with some being competent vectors (Pagès et al., 2009). The lack of investment in research into *Culicoides* taxonomy and phylogeny led to an assumption that massive outbreaks of exotic serotypes were not possible. This assumption and ensuing lack of preparation resulted in the death of millions of domestic sheep (*Ovis aries* L.) (Carpenter et al., 2009).

Most of the North American *Culicoides* species have been inadequately studied. Similar to Europe, we lack knowledge about the distribution and systematic biology of most *Culicoides*, including the vectors of BTV and EHDV (Ruder et al., 2015). In multiple regions of the United States (U.S.), both viruses are enzootic and transmission is attributed to two known vector species, *C. insignis* Lutz and *C. sonorensis* Wirth and Jones. *Culicoides insignis* has a limited distribution in the U.S. consisting of a few states in the Southeast (Blanton and Wirth, 1979; Vigil et al., 2014). *Culicoides sonorensis*, conversely, is widely distributed across North America, including parts of Mexico and southern Canada but is notably absent from the northeastern U.S. (Holbrook et al., 2000; Schmidtmann et al., 2011). One major concern is the threat of exotic serotypes of BTV being introduced to the U.S. which could lead to large outbreaks in domestic and wild ungulates. For example, BTV-8, which caused tremendous economic costs in Europe, has been detected as far west as the Caribbean (Wilson et al., 2009).

This serotype could easily be introduced into the U.S. by the movement of infected *Culicoides* into the southeastern U.S. by wind currents or hurricanes (Ducheyne et al., 2007; Wilson et al., 2009).

Considering the recent outbreaks of EHDV in the U.S. (Stevens et al., 2015), and the threat that is posed by foreign serotypes of BTV and EHDV, and viruses such as SBV and AHSV, data about the biology of North American *Culicoides* species is needed. We must clarify the taxonomy, phylogeny, and intraspecific genetic variation to understand species distributions, improve our ability to discriminate among morphologically similar species, and to recognize environmental drivers of viral transmission and vector competence (Harrup et al., 2015). Over 300 putative *Culicoides* species have been described in North America (Borkent and Grogan, 2009), but only two have been shown to be competent BTV vectors (see above) and one is suspected to be the main EHDV vector (*C. variipennis* (Coquillet); Borkent, 2005). However, other species that have not been examined thoroughly may be competent vectors and could play important roles in virus transmission (Ruder et al., 2015). In the face of impending introduction of novel viruses, molecular phylogenetic studies of North American *Culicoides* can contribute to disease risk assessment and response via outbreak source-tracking (Lau et al., 2005), identifying traits associated with vectorial capacity (Kamali et al., 2012), and detecting the presence or absence of competent vectors and susceptible hosts.

The *C. variipennis* species complex consists of three species, *C. variipennis*, *C. occidentalis* Wirth and Jones, and *C. sonorensis*. The current taxonomy is a result of multiple re-evaluations using both morphology and allozyme electrophoresis. The complex was originally described as a single species, *C. variipennis*, with five subspecies (Wirth and Jones, 1957) and subsequently the subspecies have been synonymized and/or elevated to species rank (Atchley,

1967; Downes, 1978; Holbrook et al., 2000; Wirth and Jones, 1957). In the U.S., the distribution of *Culicoides variipennis* extends from midwestern states to the East Coast, *C. sonorensis* is found from the southeastern U.S. west to the Pacific Coast, and *C. occidentalis* is restricted to southwestern and Pacific Coast states (Fig. 1; Holbrook et al., 2000). In the southeastern U.S., *C. sonorensis* and *C. variipennis* are sympatric in many of their larval habitats. In the western states, *C. occidentalis* and *C. sonorensis* co-occur in light traps but this may be due to parapatry because *C. occidentalis* has more restrictive larval habitat requirements, highly saline and alkaline bodies of water, that are outside the range of *C. sonorensis* tolerances (Holbrook et al., 2000; Schmidtmann, 2006; Schmidtmann et al., 2000; Wirth and Jones, 1957). Of the three species, *C. sonorensis* is the only known competent vector of arboviruses, but *C. variipennis* is suggested to be the main vector of EHDV despite the lack of clear, published evidence (see Discussion; Borkent, 2005; Foster et al., 1977).

The characters available to differentiate species in the *C. variipennis* complex are very limited (Holbrook et al., 2000). Females of *C. variipennis* can only be distinguished from females of *C. occidentalis* and *C. sonorensis* using the size of the third segment of the maxillary palp and its sensory pit (Fig. 3 in Holbrook et al., 2000), but females of the latter two species are indistinguishable. The only character that has been identified for differentiating *C. sonorensis* from *C. occidentalis* is the presence or absence of spicules on the aedeagus of the males, respectively (Fig. 4 Holbrook et al., 2000). However, as noted by Holbrook et al. (2000), these morphological traits can be variable and in some cases not diagnostic, which causes problems when trying to distinguish the three species in areas of sympatry. For example, the length by width ratio of the third palpal segment overlaps between *C. variipennis* and *C. sonorensis* (Holbrook et al., 2000). In these instances, Holbrook et al. (2000) suggested that additional data

were needed to assist with species identification, such as knowledge of geography, breeding habitat, and clustering of allozyme phenotypes. Thus, no consistent method exists to distinguish the three species. These complications cause problems for vector surveillance and determination of the ranges of each species as sample processing times can be significant and the confidence in species identification is low for non-*Culicoides* experts.

My objective for this study was to use a multi-locus molecular phylogenetic approach to test the hypothesis that the *C. variipennis* complex consists of three distinct species in the U.S. I collected all three species from across the U.S., sequenced multiple mitochondrial and nuclear DNA loci, and used tree-building methods, coalescent theory-based approaches, population genetic analyses, and demographic statistics to better understand the phylogenetic relationships and diversification of the *C. variipennis* species complex. My ultimate goal was to provide additional data to solidify the foundation of species identification and range estimates of *Culicoides* in North America which will help improve not just our understanding of the evolution of this taxonomic group, but also to inform and improve *Culicoides*-borne disease risk assessments and disease management.

Materials and Methods

Sample collection, DNA extraction, PCR amplification, and DNA sequencing

Between January 2011 and November 2014, I, and other vector or disease biologists, collected 291 *Culicoides* from multiple locations in 25 states across the U.S. in an attempt to obtain samples throughout the ranges of the three species within the *C. variipennis* complex (Fig. 1; Appendix 1). Insects were collected using either CDC mini light traps (Bioquip, U.S.A) or New Jersey traps (Bioquip, U.S.A) both baited with CO_2 and UV light and located

approximately 2 m above the ground. The traps were set from 2 hours before sunset to 2 hours after sunrise. Trap catches were immediately stored in absolute ethanol or at -80°C until sorting. *Culicoides* were sorted from by-catch under a dissecting microscope. The *C. variipennis* complex, and outgroup species, were identified based on wing patterns (Wirth et al., 1985). I identified the three species within the *C. variipennis* complex using the morphological characters or geographic locations outlined in Holbrook et al. (2000). I also included four individuals from the captive colonies at the USDA-Agricultural Research Service Laboratory in Manhattan, Kansas as known references of *C. sonorensis* individuals with known vector competence. DNA was extracted from specimens using the DNeasy Blood and Tissue Kit (Qiagen, Germany). Each insect was placed in 300 µL of buffer ATL, either whole or separated into head/thorax and abdomen. Twenty µL of proteinase K was added to buffer ATL and the insects were incubated at 56°C overnight. Following incubation, the second day extraction steps were carried out on a Qiacube (Qiagen, Germany) using the purification protocol for forensic casework samples under the Qiagen Investigator Kit option provided by the manufacturer. The elution volume was set at 50 µL and extracts were stored at -20°C until downstream processes.

I amplified and sequenced DNA from four loci, two mitochondrial and two nuclear. The mitochondrial loci included 733 base pairs (bp) of the cytochrome oxidase subunit one gene (COI) and 773 bp of the cytochrome oxidase subunit two gene (COII). The nuclear loci consisted of 679 bp of the carbamoyl phosphate synthetase region of the CAD (rudimentary) gene (CAD), and 440 bp of triose phosphate isomerase gene (TPI). The PCR conditions are provided in Appendix 2. PCR success was evaluated using 2% agarose gels stained with ethidium bromide. PCR products were purified using ExoSAP (Affymetrix, U.S.). Cycle sequencing in both directions was completed using BigDye version 3.1 (ThermoFisher, U.S.). Excess BigDye was

removed using sephadex (Sigma, U.S.) in Prepease spin columns (Affymetrix, U.S.). Sequence data were collected using Applied Biosystems Genetic Analyzers 3130xl and 3500xl (ThermoFisher, U.S.). Raw sequences were manually checked and edited in SEQUENCHER v.5.2.4 (GeneCodes, U.S.) or GENEIOUS v7.1.5 (Biomatters, U.S.). Sequences were aligned using either the MAFFT (Katoh et al., 2005; Katoh et al., 2002) or CLUSTALW (Larkin et al., 2007) multiple alignment plug-ins in GENEIOUS v7.1.5. The datasets for each of the two nuclear loci contained heterozygotes. Ambiguous data in DNA sequences can result in biased tree topologies and branch lengths (Lemmon et al., 2009) so I used the software PHASE (Stephens and Scheet, 2005) to estimate all haplotypes for each individual. I also downloaded 15 *variipennis* complex COI sequences collected in Canada from the Bar Code of Life Database (BOLD (Ratnasingham and Hebert, 2007); 12 from British Columbia and three from Saskatchewan; Accession #s CFWIG804, CNGRD391, CNGRD743, CNGRF623, GBDP18067-GBDP18074, NGAAA506, NGAAA946, NGAAC1467, NGAAD616, NGAAD660, NGAAE438, NGAAE509, NGAAF349, NGAAF427, NGAAF436, NGNAK126, NGNAR328). For gene tree analyses I collapsed the DNA sequences into haplotypes using ALTER (Glez-Peña et al., 2010). Molecular diversity indices for each gene (e.g., %AT and %GC, transition/transversion ratio, number of polymorphic sites, haplotype diversity, and nucleotide diversity (π)) were calculated using ARLEQUIN v3.5 (Excoffier and Lischer, 2010).

Phylogenetic analyses

I used PARTITIONFINDER V1.1 (Lanfear et al., 2012) to identify the nucleotide substitution models that best fit the datasets for each gene. The best fit models were: COI (GTR+I+G or TVM+I+G); COII (HKY+I+G); CAD (GTR+I+G); TPI (GTR+I+G or SYM+I+G). Distinguishing between two models for the COI and TPI loci was not possible using typical

model selection criteria (AICc, BIC, and likelihood ratio) so I implemented the GTR model as it is the most readily available model in analysis software where TVM or SYM are often absent (e.g., GARLI v2.0 (Zwickl, 2006)).

Using the best-fit models (GTR+I+G for all loci except COII for which I used HKY+I+G), I constructed gene trees using both maximum likelihood (ML) and Bayesian approaches. The ML trees were generated using GARLI v2.0 implemented on CIPRES Science Gateway (Miller et al., 2010a). I ran 1000 bootstrap replicates. A majority-rule consensus tree was generated from the output files using the CONSENSE program (PHYLIP; Felsenstein, 1993) also implemented on CIPRES. Bayesian trees were constructed in the MRBAYES v3.2 plug-in (Huelsenbeck and Ronquist, 2001) for GENEIOUS. I ran a Metropolis-coupled Monte Carlo Markov Chain (MC^3) of 1,100,000 iterations with 100,000 burn-in replicates and unconstrained branch lengths. Convergence was evaluated in GENEIOUS and majority-rule consensus trees with branch posterior probabilities were estimated with TREEANNOTATOR v2.3 (Bouckaert et al., 2014). The outgroups for the gene trees consisted of the species *C. butleri* Wirth and Hubert, *C. crepuscularis* Malloch, *C. defoliarti* Atchley and Wirth, and, *C.* (*Selfia*) sp. Khalaf. Not all outgroups were included in each tree; inclusion was based on whether I successfully sequenced DNA for a species for a particular locus (Appendix 1). The *C. variipennis* complex is currently placed in the *Monoculicoides* subgenus. To test the monophyly of this taxonomic grouping, I also downloaded COI sequences from Palearctic members of the *Monoculicoides* (*C. nubeculosos* (Meigen), *C. riethi* Kieffer, and *C. stigma* (Meigen)) from BOLD and included these in the phylogenetic analyses (Accession #: KF178272, JQ620195, KF178281).

Using a total evidence approach from the four loci that I evaluated, I estimated the species tree using *BEAST (Bouckaert et al., 2014; Heled and Drummond, 2010). This approach

utilizes a Bayesian framework and the multi-species coalescent theory to reconcile the phylogenetic signals across a multilocus dataset into a single species tree. *BEAST accounts for uncertainty in gene tree topologies caused by factors such as incomplete lineage sorting in recently diverged species. I used *C. crepuscularis*, *C. defoliarti*, and *C.* (*Selfia*) sp. as outgroups in this analysis. The models implemented assumed a strict molecular clock, constant population size, birth-death tree model, and the MCMC was run for 400,000,000 iterations. Of the genes that I included in the data set, only COI has an estimated per lineage molecular clock rate in insects. The current standard rate used for insects is 1.15% per million years (Brower, 1994) and was estimated from butterflies; the other rate was estimated more recently in a lineage of beetles and is 1.77% per million years (Papadopoulou et al., 2010). To determine if these two different rates had notable effects on the tree topology for *Culicoides,* I ran *BEAST with both rates and then estimated rates for the three other loci under both scenarios. All phylogenetic trees were visualized and edited using FIGTREE v1.4 (Rambaut, 2014).

Very recent speciation events can lead to a lack of monophyly in reconstructed phylogenies due to reticulate evolution and violation of the assumptions of bifurcating evolutionary lineages, which is common at the interface of phylogenetics and population genetics (Posada and Crandall, 2001). Variation in the morphological characters used to study the *C. variipennis* complex is mostly homogeneous suggesting very recent divergence. Thus, I employed median-joining (M-J) networks (Bandelt et al., 1999) constructed in POPART (http://popart.otago.ac.nz) for each locus to better understand the relationships of highly similar haplotypes. All outgroups were excluded from the network analysis, but I included redundant haplotypes.

I calculated two genetic distances, Kimura-2 parameter (K2P) and *p*-distance, to evaluate the levels of differentiation between clades in the phylogenetic trees, the haplogroups in the M-J networks, and the genetic clusters defined below. I chose the K2P distance because it has been used to evaluate differentiation between Palearctic *Culicoides* COI sequences and gave me a frame of reference for interspecific and intraspecific genetic distances (Ander et al., 2013). To evaluate if the K2P distance was biased I also calculated the *p*-distance to determine if the distances conflicted; the *p*-distance has been recommended as more appropriate for use with the COI locus (Collins and Cruickshank, 2013). These distances were calculated in MEGA7 (Kumar et al., 2016).

Population genetic analyses

Given that there was limited genetic divergence among individuals in clade I (see Results), I investigated intraspecific processes such as gene flow and selection, as explanations for homogeneity among samples. Initially I tested for the presence of population structure in my dataset. I estimated population structure using the spatial clustering of individuals option in BAPS v6.0 (Cheng et al., 2013; Corander et al., 2008). BAPS employs a Bayesian clustering algorithm that assigns individuals to population clusters based on shared allele frequencies. Incorporating spatial data allows BAPS more resolution to identify fine scale population structure that might otherwise be undetected. I only included individuals identified as belonging to Clade I in the phylogenetic trees (see Results) using three loci, COI, CAD, and TPI. The COII locus is a highly conserved region and lacked sufficient diversity for population genetic analyses. I ran BAPS with the maximum values of *k* set at 5, 10, 20, and 30 and I ran 5 replicates of each *k*.

The demographic history of Clade I is of biological interest considering its membership includes competent pathogen vectors (e.g., *C. sonorensis*) and my results showed little phylogenetic resolution between *C. sonorensis* and *C. variipennis*. To further investigate the evolutionary history of this group, I calculated statistics that measure selection and historical demographics and used these metrics to infer factors that may have contributed to patterns of genetic diversification. Using ARLEQUIN, I calculated Tajima's *D*, Fu's *Fs*, and the mismatch distribution (Fu, 1997; Rogers and Harpending, 1992; Slatkin and Hudson, 1991; Tajima, 1989a, b). I examined these statistics to determine the neutrality of the loci while accounting for historical demography. A significant negative value of Tajima's *D* means a rapidly expanding population whereas a positive value suggests a recent genetic bottleneck. Fu's *Fs* is the most sensitive to demographic changes of the three statistics and a significant negative value is associated with recent demographic expansion. The null hypothesis for *D* and *Fs* when considering historical demography is stability, whereas significant test suggests a recent expansion. Using the mismatch distribution, I tested for recent population expansion versus and classified a population as stable if the distribution of the observed pairwise differences between DNA sequences was multimodal; whereas a smooth bell curve of these differences was classified as a recent expansion. I tested for statistical significance of mismatches between expected and observed distributions using two approaches, sum of squared deviations (SSD) and the raggedness index (*r*). The null hypothesis for the mismatch distribution is demographic expansion.

Bayesian skyline plots (BSP) illustrate estimates of dates for demographic changes leading to hypotheses about historical biogeography of this group; BSPs can provide insight into the timing of diversification which can support or refute the current taxonomy. In order to put a

timeframe on historical demographic events and to test further the hypotheses of recent population expansion of mtDNA, I estimated a BSP using BEAST v2.3 (Bouckaert et al., 2014; Drummond et al., 2005) on the COI data for the clade I. The skyline plot shows estimates of effective population size (N_e) over time and these estimates were generated using coalescent theory and application of a molecular clock. I limited the BSP analysis to the COI locus because it was the only one to which I could apply a molecular clock as there are not estimates for the other loci. I ran the MCMC for 50,000,000 iterations with 10% burn-in, a strict molecular clock, and the GTR+I+G substitution model. I calibrated the molecular clock using the rates I employed in the species tree, 1.15% and 1.77% per million years per lineage (see above). The BSP output includes a plot of the mean estimated N_e across time plus confidence intervals.

Results

DNA sequencing

Total sample sizes of individuals in the groups in the *C. variipennis* complex that I sequenced ranged from 164 to 242 and the number of haplotypes for each locus ranged from 53 to 199 (Table 1 and 2). Across all loci the COII had the lowest diversity with the fewest haplotypes and the fewest polymorphic sites. The two nuclear loci were more diverse than the mtDNA and the mtDNA loci were highly A/T rich (Table 1). I found 87 heterozygous individuals for the CAD locus and 153 heterozygous individuals for the TPI locus.

Phylogenetic analyses

The ML and Bayesian trees for each locus were virtually identical; all the major clades with high branch support matched and any differences in topology were at the tips of branches where relationships were unresolved. Because of the high degree of overall genetic similarity, I

present only the Bayesian trees (Figs. 2 and 3). The subgenus *Monoculicoides* was monophyletic with high branch support in the COI tree. All of the different gene trees had similar topologies with high branch support for a single, large clade that included all of the *C. variipennis* complex individuals (Figs. 2 and 3). This single clade was further split into two clades. The first and largest clade (Clade I) included all individuals morphologically identified as *C. sonorensis* (including the individuals from the USDA captive colony), *C. variipennis*, and female individuals from western states where species identification was impossible due to sympatry of *C. occidentalis* and *C. sonorensis* (Table 2). Within clade I, the branch support was low and relationships were poorly resolved.

A second, well-supported clade (Clade II) contained the males identified as *C. occidentalis* and females collected from western states, mostly California. In the mtDNA trees there was third poorly supported clade (Clade III) that comprised females sampled from across the western U.S. (Fig. 2; Table 3). Clade I demonstrates that *C. variipennis* and *C. sonorensis* from states east of the Rocky Mountains were paraphyletic. The only currently recognized species within the *variipennis* complex that was monophyletic across all four loci was *C. occidentalis.*

The Bayesian estimated species tree from *BEAST was similar to the individual gene trees (Fig. 4). The tree had a clade with posterior probability support of 0.82 that included all of the *variipennis* complex samples. Within this clade there was high support for two groups, one that was equivalent to *C. occidentalis* (Clades II and III) and one that included all samples in the *C. sonorensis/C. variipennis* clade (Clade I) in the gene trees.

The haplotype networks had higher resolving power than the phylogenetic trees for understanding relationships among haplotypes at each locus (Figs. 4 and 5, Table 2). The results from the networks were similar to the gene trees in that there were two clearly defined haplogroups, one correlating to Clade I and a second to Clade II, reflecting those individuals collected from western states. The mtDNA networks had additional haplogroups with individuals collected in western states and western Canada that matched clade III (Fig. 4). The mtDNA loci were the least diverse and had the most redundant haplotypes. Both mtDNA loci had haplotypes that occurred in a starburst pattern which is indicative of a recent population expansion. Both nDNA loci had more haplotypes and more singletons than the mtDNA loci. Within the *C. sonorensis*/*C. variipennis* haplogroup (group A in all networks) some geographic substructuring existed with haplogroups located in general geographic regions (e.g., west, southeast, Midwest/east); however, many haplotypes were broadly distributed geographically (Table 2). All loci had haplotypes that were shared among individuals that ranged from Rocky Mountain States to the Atlantic Coast.

There were divergent mtDNA haplotypes that did not group with *C. sonorensis*/*C. variipennis* (Clade I) or *C. occidentalis* (Clade II). Haplogroups COI-C and COI-D (Fig. 4) included individuals that were all collected from the western U.S. and fell into the poorly supported mtDNA clade III (Fig. 5). These samples were females with the swollen maxillary palp character state associated with either *C. sonorensis* or *C. occidentalis* (Table 2). Haplotypes from COI-D were also divergent in the COII (COII-C) and CAD (CAD-C) networks. The number of base pairs separating these haplotypes from other haplogroups ranged from four to ten. These samples did not amplify for the TPI gene. There were two individuals that belonged to the divergent COI-C haplogroup. In the phylogenetic trees, these sequences were found in clade

II. These were females from California which could be identified as either *C. sonorensis* or *C. occidentalis* using morphology. The groupings of these samples for the CAD locus were not consistent. One sample belonged to clade I, the CAD-A haplogroup, and the CAD-2 BAPS cluster (see below), while the other sample grouped with Clade II, CAD-A haplogroup, and CAD-2 cluster. For the TPI gene, the pattern of these two individuals was even more different. The first fell into TPI Clade I, TPI-A haplogroup, and TPI- cluster. The second sample fell into TPI clade II, and the TPI- haplogroup.

Population genetic analyses

The clustering results from BAPS varied based on the locus analyzed (Fig. 4, 5; Table 2). There was only one (K = 1) genetic cluster identified in the COI locus, two (K = 2) in the CAD locus (CAD-1 and CAD-2), and three (K = 3) in the TPI locus (TPI-1, TPI-2, and TPI3) (Figs. 4 and 5, Table 2). The two CAD clusters consisted of one (CAD-1) that ranged across the continental U.S. and included individuals with both the swollen and slender maxillary palp character states, and the second (CAD-2) included individuals from the eastern U.S. with slender maxillary palps. TP1-1 consisted of individuals from the eastern U.S. and were identified morphologically as *C. variipennis* with the slender maxillary palps. TP-3 included individuals from the western and southeastern U.S. and exhibited the maxillary palp characteristic of *C. sonorensis* and *C. occidentalis*. TPI-2 was a genetic cluster that included individuals with maxillary palp characteristic states consistent with both *C. sonorensis* (swollen palps) and *C. variipennis* (slender palps), and that ranged from the Rocky Mountains to the Atlantic Coast. The TPI-1 and CAD-2 clusters included the same individuals, as did CAD-2 and TPI-2 and TPI-3.

I detected a signal of recent population expansion in all loci. The COI locus had a non-significant negative values of Tajima's D (D = -1.53, P = 0.027) but significant Fs = -24.86 (P = 0.00), and a mismatch distribution r (P = 0.4) and SSD (P = 0.32) that were not statistically significantly different than the null hypotheses (Fig. 6). A signal of population expansion was also evident in the CAD [(D = -0.21, P = 0.5), Fs = -23.62 (P = 0.004)); mismatch distribution r (P = 0.98); SSD (P = 0.80)] and TPI loci [D = -0.25 (P = 0.66), Fs = -24.22 (P = 0.001)), and mismatch distribution r (P = 0.27); SSD (P = 0.002)]. The observed distribution of base pair changes for all loci did not significantly differ from the expected for any locus which suggests recent population expansion (Fig.6). The COI BSP demonstrated that the maternal lineage N_e of clade I was stable for the last 12,000 years. I detected a slow, steady decline between 2,000 (molecular clock = 1.15%) and 1,500 (molecular clock = 1.77%) years before present (ybp; Fig. 8) with a rapid population expansion during the last century.

The mean genetic distances within and between haplogroups and clusters was different for each locus. The CAD locus showed the highest divergence (Table 3). The mean K2P distances between major clades (e.g., outgroups and *variipennis* complex) were: COI = 0.18; COII = 0.16; CAD = 0.22; and TPI = 0.11. Within the *Monoculicoides* the mean K2P distance was COI = 0.13. Within the *variipennis* complex the mean K2P distances were: COI = 0.03; COII = 0.02; CAD = 0.06; and TPI = 0.02. The *p*-distances did not differ from the K2P distances.

Discussion

My findings draw into question the currently accepted taxonomy of the *C. variipennis* complex. Considering that taxonomy is an important base upon which many other fields of

biology build (Wheeler, 2004; Wheeler et al., 2004), my objective was to evaluate the current taxonomic hypothesis within the *C. variipennis* species complex using molecular phylogenetic and population genetic approaches. The current hypothesis states that three species, *C. occidentalis*, *C. sonorensis*, and *C. variipennis*, form the *C. variipennis* complex and that these species can be distinguished using morphological traits, one for females and one for males (Holbrook et al., 2000). For some specimens, however, these traits are indeterminate and in these cases knowledge of the breeding habitat is invoked to achieve species identification. However, Holbrook et al. (2000) acknowledged that in many regions across the U.S. multiple species share the same breeding habitats. This sympatry, the questionable morphological characters, and lack of phylogenetic resolution across four genetic loci demonstrated the need for a new taxonomic reevaluation of this group.

Phylogeny and population structure

Molecular methods have contributed to systematics by adding additional data that provide independent tests of taxonomic hypotheses. In many instances, molecular data support the established species designations (e.g., Patterson et al., 1993), but, in other cases, the data reveal a deeper complexity that draws into question current classifications (e.g, Hedges and Sibley, 1994). In cases of very recent diversification the complexities uncovered can confound species delimitation because of the lack of monophyly caused by incomplete lineage sorting (*sensu* Avise et al., 1983). In these cases, an integrative taxonomic approach should be utilized that includes morphology, ecology, phylogenetics, but also population genetic analyses.

The phylogenetic relationships of the DNA sequences, haplotype networks, and cluster analyses of the *C. variipennis* complex do not directly correspond to the three species proposed

by Holbrook et al. (2000) (Table 2; Figs. 2, 3, 4, and 5). None of the four genes that I examined supported *C. sonorensis* and *C. variipennis* as two distinct, monophyletic groups. Through the use of haplotype networks and Bayesian clustering, I showed that different genes demonstrated differing abilities to distinguish genetic clusters. The mtDNA loci lacked resolution at both the phylogenetic and population genetic levels. I detected weak genetic structure in nDNA loci that was loosely tied to geography (Table 2; Fig. 6). There were multiple clusters in both CAD and TPI and each had one cluster that included individuals morphologically identified as *C. variipennis* collected within the currently recognized range of this species. However, both nuclear loci also had clusters that consisted of individuals identified as both *C. variipennis* and *C. sonorensis* that ranged from coast to coast in the U.S. The average K2P distance between any of the groups within the *C. varipennis* complex was 0.033 (range: 0.027 – 0.037; Table 3). This distance was less than the minimum intraspecific K2P distance for Palearctic *Culicoides* species (range: 0.13 – 0.31; Ander et al., 2013). The lack of divergence, inconsistent species identification using the maxillary palps, and population level genetic structuring of the individual sequences does not support the current *C. sonorensis* and *C. variipennis* species' designations.

In all analyses I identified one genetically distinct and monophyletic group with high statistical support (Clade II) that corresponded morphologically and geographically to *C. occidentalis.* This clade contained males that lacked spicules on the aedeagus, the diagnostic morphological character state identified by Holbrook et al. (2000). These individuals also formed a unique haplogroup in each of the haplotype networks. The K2P distances separating the B haplogroups from others in the *C. variipennis* complex across all loci ranged from 0.022 to 0.064. The mean COI distance was 0.028, still far below distances between well-defined species (Ander et al., 2013). Individuals identified as *C. occidentalis* occur in larval habitats that are

highly saline (e.g., playa lakes) and outside the tolerance of individuals in Clade I (Schmidtmann, 2006; Schmidtmann et al., 2000). Despite the low genetic distances, the monophyly of sequences across all four loci, the spiculate aedeagus character state, and the unique breeding habitats all support consideration of *C. occidentalis* as an independently evolving lineage.

I found some mtDNA sequences from the western U.S. that did not group with either Clades I or II, nor did they group with the major haplogroups associated with these clades. These sequences came from distant areas in the western states ranging from California to as far east as Wyoming and New Mexico, and as far north as British Columbia. All of the BOLD COI sequences from British Columbia belonged to these clades and haplogroups. These sequences came from individuals were originally identified as *C. sonorensis*, however, they did not group with Clade I individuals in any of my analyses. The sample sizes of these sequences, and also of *C. occidentalis*, are not large enough to draw inferences about the evolutionary history of these two groups in the western U.S. I have, however, identified the need for a more thorough sampling of western states to delineate the true ranges of these unique genetic groups which will help to elucidate the origins, possibility of cryptic species, or identify the need for more thorough taxon sampling.

Two previous studies have suggested that *C. sonorensis* and *C. variipennis* are more closely related to each other than they are to *C. occidentalis.* Tabachnick (1992) used multiple allozyme loci and a UPGMA tree and found that *C. sonorensis* and *C. variipennis* grouped together with *C. occidentalis* as a distinct, divergent cluster. Shults et al. (2016) evaluated the morphology of the pupa of the *Monoculicoides.* These authors found a morphological pattern similar to my molecular data; the morphology of *C. sonorensis* and *C. variipennis* pupa were

more similar to each other than was the morphology of *C. occidentalis* to the other two. However, they still found differences between *C. sonorensis* and *C. variipennis* but these were less pronounced than between *C. occidentalis* and the others. These authors did not, however, analyze the data in a phylogenetic context. It should also be noted that this study used laboratory-raised *C. sonorensis* specimens and not wild types. I found individuals from the laboratory colonies at USDA-ARS (n = 4) to be genetically identical at mtDNA loci (all sharing a single haplotype), which could be indicative of either inbreeding or selection associated with domestication. The morphology of the laboratory specimens may have been influenced by either one of these pressures and the morphological differences found by Shults et al. (2016) may not be indicative of evolutionary history or natural variation of *C. sonorensis*. A similar bottleneck of morphological variation has been observed in *Aedes aegypti* by Schneider et al. (2011) and they cautioned against extrapolating inferences from laboratory colonies to wild populations as the data can be biased by artificial processes.

Species exposed to high environmental variation tend to be more phenotypically malleable than species maintained in constant environments (Atchley, 1971; Levins, 1965, 1969). Phenotypic plasticity is common in many broad-ranging insect taxa and can muddle morphological species identification (Moczek, 2010), which may explain the variation observed in maxillary palp morphometry of the *C. variipennis* species complex. In fact, in the original description of this species complex, Wirth and Jones (1957) commented on the inconsistency of the morphological traits, which they associated with environmental variability. This environmental influence hypothesis was supported by Atchley (1971) who determined that exposure of larval *Culicoides* species in the subgenus *Selfia* to certain environmental conditions can modify adult morphology. A pertinent example of phenotypic plasticity and its influence on

species identification is the Triatominae (Hemiptera: Reduviidae), the vectors of the causative agent of Chagas disease, *Trypanosoma cruzi* (Steverding, 2014). The taxonomy of this group has been in flux and the identification of vector species problematic. The obscure morphology-based species identification has been attributed to phenotypic plasticity caused by exposure to environmental variability, and is not tied to concomitant mutation in DNA sequences (Dujardin et al., 2009; Schofield and Galvão, 2009). Similar to the Triatomine, I have seen very little genetic divergence within the *C. variipennis* complex which suggests that much of the morphological variation that has been observed in this group may be attributed to differences in habitats. My results highlight a need for further investigation using both laboratory experiments with wild types and ecological studies to assess the factors that might contribute to the variability in and diversification of the *C. variipennis* complex.

Historical demography

The historical biogeography of *Culcioides* species in North America is largely unknown but knowledge of it can help understand the complexities of recently diverged groups. Wirth and Jones (1957) suggested that the *C. variipennis* complex may have been widespread as far back as the Pleistocene. The COI BSP confirmed that there was a stable, large N_e dating back to 12,000 ybp. Starting around 2,000 ybp I saw a slow decline in N_e with a dramatic increase during the last century (in the BSP and the demographic statistics). I speculate that one reason for the decline could have been a selective sweep on the mtDNA genome. Bazin et al. (2006) noted that mtDNA in insects was, on average, less diverse than nDNA. This reduced mtDNA diversity has been associated with selective sweeps driven by the presence of a *Wolbachia* parasite (Hurst and Jiggins, 2005). *Wolbachia* occurs with high prevalence in many insect taxa: up to 66% of species are thought to be infected and prevalence can be up to 90% within individual species

(Hilgenboecker et al., 2008). Following the sweep, selection may have been relaxed as drift is the dominant force in small populations, and mutations built up as the populations expanded due to human-driven land use changes. During the last two centuries in the U.S., humans have converted large tracts of land to agriculture, which increased water in previously arid regions, introduced livestock, and opened up forests creating more edge habitat that favors large ungulates such as white-tailed deer (Rooney and Waller, 2003). These agricultural impacts, combined with increased temperatures due to climate change, have also contributed to increased eutrophication of water bodies (Downing, 2014). All of these factors have increased the number of breeding habitats and hosts for *Culicoides*, lengthened the *Culicoides* biting and breeding seasons, and increased the speed of the life cycle resulting in more generations of insects produced per year (Bishop et al., 1996; Elbers et al., 2015; Vaughan and Turner, 1987). Taken together, these environmental factors could explain the rapid increase in effective population size of the *variipennis* complex, which led to occupancy and isolation of diverse habitats resulting in the morphological variation we see today.

Implications: Taxonomy, pathogen transmission, disease ecology, and management

The uncertain taxonomy of the *C. variipennis* complex has huge implications for understanding BTV and EHDV transmission in North America. The lack of BTV transmission in the northeastern U.S. has been attributed to the absence of the known vector species, *C. sonorensis* (Tabachnick, 1996; Tabachnick and Holbrook, 1992). The four genetic loci I analyzed supported distinction of only two species in the *C. variipennis* complex: *C. occidentalis*, and another unnamed species that occurs across the continental U.S. The genetic similarity and the limited population structure in this wide-ranging clade indicate that the lack of transmission of BTV in the northeastern states is not due to absence of the potential vector, but is

likely due to population differences in vectorial capacity or unidentified ecological factors. In the Northeast, the *C. variipennis* complex is known to occur in lower densities and experiences harsher winters than in other parts of its range (Holbrook et al., 1996). Considering that vector abundance is one aspect of vectorial capacity (Garrett-Jones, 1964), the lower population sizes in this portion of the U.S. could be a limiting factor for transmission. Kilpatrick et al. (2010) demonstrated that different populations within two species of mosquitoes, *Culex pipiens* L. and *Cx. restuans* Theobald, have variable levels of vector competence driven by the environment and population genetic factors. My data support the idea that vector competence in *Culicoides* populations may be variable as it is in mosquitoes.

Ambiguous taxonomy can lead to questionable interpretations of pathogen vector status. *Culicoides variipennis* is the current hypothesized vector of EHDV in North America (Borkent, 2005). The data that are cited as proof of vector competence are from Foster et al. (1977) who demonstrated transmission of EHDV by what was identified as *C. variipennis* from Kentucky. This study pre-dated Holbrook et al. (2000) and the current taxonomy within the *C. variipennis* complex. At the time of the Foster et al. (1977) study, the *C. variipennis* complex was considered a single species, *C variipennis*. Both *C. variipennis* and *C. sonorensis*, as defined by Holbrook et al. (2000), occur in Kentucky. None of the specimens used in the Foster et al. (1977) study are available for re-examination so it is not clear which species was used in their study leaving the question of vector competence open. Transmission of EHDV only by *C. variipennis* has been drawn into question by recent outbreaks of EHDV outside this species' currently known range. These outbreaks occurred in the Rocky Mountain States and as far west as Oregon in an endangered population of the Columbia white-tailed deer (*Odocoileus virginianus leucurus* (Douglas)) (ProMED-mail, 2012; Stevens et al., 2015). Evidence from both current and

historical EHDV outbreaks is further evidence that: 1) *C. sonorensis* and *C. variipennis* are not different species, but rather one single group that ranges from coast-to-coast in the U.S.; and 2) that the EHDV vector status of *C. variipennis* is unclear at best and its likely not the only vector of EHDV in the U.S. (Ruder et al., 2015).

The molecular data I collected and analyzed do not support the current taxonomy of the *C. variipennis* complex. I suggest that a taxonomic reevaluation to include a more extensive molecular dataset, broader geographic sampling including Canada, U.S., and Mexico, and a re-examination of morphological characters must occur. Soon, very large phylogenomic and population genomic datasets will be cheaper and easier to generate. A fully assembled and annotated *C. sonorensis* genome will also soon be available and genome-wide association studies for vector competence for BTV and AHSV will be undertaken (Nayduch et al., 2014). The *C. sonorensis* genome will also be useful for rigorous phylogenetic and population inferences regarding evolution, vector competence, immunology, and ecology of *Culicoides* species and the pathogens they transmit. In particular, applying whole genomes in a similar fashion to this study should shed light on differences in transmission in different regions, allow further refinement of taxonomic assignments, and contribute to our understanding of the transmission of *Culicoides*-borne pathogens worldwide.

Table 1- Molecular diversity indices and total individuals collected (n) for the four genetic loci sequenced from the *C. variipennis* complex collected across the continental U.S. The clades in the phylogenetic trees from Figures 2 and 3 are represented by Roman numerals. The number of base pairs for each locus, number of haplotypes, percent A and T (%A/T) and percent G and C (%G/C), total number of polymorphic sites, transition to transversion ratio, haplotype diversity (*H*), and nucleotide diversity (π) are presented.

Locus	Clade	base pairs	n	haplotypes	%A/T	%G/C	Polymorphic sites	TI/TV	*H*	π
COI	I	733	191	70	68.27	31.73	72	2.46	5.9	0.01
	II/III	733	34	19	68.67	31.33	35	2.72	9.6	0.02
COII	I	773	161	40	75.06	24.94	44	3.18	3.48	0.005
	II/III	773	14	13	75.48	24.51	28	6	9.03	0.01
CAD	I	679	228	181	60.53	39.46	170	1.6	26.42	0.04
	II	679	18	17	60.31	39.69	63	1.5	17.92	0.03
TPI	I	440	268	104	55.81	44.19	50	1.48	8.8	0.02
	II	440	17	7	55.92	44.08	8	3.5	2.74	0.006

Table 2- Summary of the clades, haplogroups, and genetic clusters per locus from the *Culicoides variipennis* complex collected across the continental U.S. The number of individuals (n), U.S. and Canadian states from which the individual *Culicoides* were collected, morphological species identification (morphospecies), and sex of the samples are provided.

Locus	Clade	Haplogroup	Cluster	n	States	Moprhospecies	sex
COI	I	COI-A		115	AL, AZ, CO, ID, LA, MT, NC, ND, NE, NM, NV, SC, TX, WY	*sonorensis*	female
	I	COI-A		75	IL, IN, LA, MI, MO, NC, NJ, OH, PA, SC, TX	*variipennis*	female
	II	COI-B		6	CA	*sonorenesis* or *occidentalis*	female
	II	COI-B		5	CA	*occidentalis*	male
	II	COI-C		2	CA	*sonorenesis* or *occidentalis*	female
	III	COI-D		21	BC, CA, CO, ID, WY	*sonorenesis* or *occidentalis*	female
COII	I	COII-A		90	AL, AZ, CO, ID, LA, MT, NC, ND, NE, NM, NV, SC, TX, WY	*sonorensis*	female
	I	COII-A		70	IN, LA, MI, MO, NC, NJ, OH, PA, SC, TX	*variipennis*	female
	II	COII-B		2	CA, NV	*sonorenesis* or *occidentalis*	female
	II			5	CA	*occidentalis*	male
	III	COII-C		7	CA, ID, NM, WY	*sonorenesis* or *occidentalis*	female
CAD	I	CAD-A	CAD-1	123	AL, AZ, CA, CO, ID, LA,MT, NC, ND, NM, NV, SC, TX, VA, WY	*sonorensis*	female
	I	CAD-A	CAD-1	9	LA*, MI, NJ, TX*	*variipennis*	female
	I	CAD-A	CAD-2	92	AL, IN, LA, MO, NC, NJ, OH, PA, SC, TX	*variipennis*	female
	II	CAD-B		15	CA, CO, NM	*sonorenesis* or *occidentalis*	female
	II			3	CA	*occidentalis*	male
TPI	I	TPI-A	TPI-1	37	IL, IN, KS, LA, MO, NC, NJ, PA, SC, TX	*variipennis*	female
	I	TPI-A	TPI-2	33	AL, CO, KS, MT, ND, WY	*sonorensis*	female
	I	TPI-A	TPI-2	11	MI, NJ	*variipennis*	female
	I	TPI-A	TPI-3	70	AZ, CA, CO, ID, KS, LA, NC, NM, NE, NV, SC, TX	*sonorensis*	female
	II	TPI-B		8	CA, ID, NV	*sonorensis* or *occidentalis*	female
	II	TPI-B		5	CA	*occidentalis*	male

Table 3- Pairwise genetic distance matrices for each locus, COI, COII, CAD, and TPI sequenced from *Culicoides variipennis* complex collected across the continental U.S. The lower matrix is the Kimura-2 parameter and the upper matrix is the p-distance. The distances calculated within the *C. variipennis* complex were between clades (Roman numerals), haplogroups (capital letters), and BAPS clusters (Arabic numerals).

	COI-A	COI-B	COI-C	COI-D	*C. riethi*	*C. stigma*	*C. nubeculosus*	*C. (Selfia) sp.*	*C. defoliarti*	*C. butleri*	*C. crepuscularis*
COI-A		0.031	0.034	0.034	0.132	0.136	0.160	0.155	0.183	0.249	0.151
COI-B	0.032		0.027	0.027	0.135	0.144	0.154	0.160	0.177	0.253	0.162
COI-C	0.036	0.027		0.036	0.137	0.140	0.165	0.159	0.172	0.245	0.145
COI-D	0.036	0.028	0.037		0.139	0.134	0.163	0.151	0.188	0.247	0.153
C. riethi	0.145	0.149	0.152	0.154		0.142	0.130	0.161	0.192	0.202	0.178
C. stigma	0.150	0.160	0.156	0.148	0.158		0.184	0.142	0.174	0.227	0.178
C. nubeculosus	0.180	0.173	0.186	0.184	0.143	0.212		0.159	0.171	0.218	0.184
C. (Selfia) sp.	0.174	0.181	0.178	0.169	0.182	0.158	0.179		0.167	0.235	0.177
C. defoliarti	0.210	0.202	0.196	0.217	0.222	0.198	0.194	0.189		0.236	0.186
C. butleri	0.303	0.309	0.297	0.300	0.237	0.270	0.258	0.282	0.284		0.248
C. crepuscularis	0.169	0.182	0.161	0.171	0.204	0.204	0.212	0.202	0.214	0.302	

	COII-A	COII-C	COII-B	*C. (Selfia) sp.*	*C. defoliarti*	*C. crepuscularis*
COII-A		0.022	0.022	0.144	0.171	0.201
COII-C	0.022		0.023	0.137	0.169	0.188
COII-B	0.022	0.024		0.140	0.166	0.194
C. (Selfia) sp.	0.161	0.152	0.156		0.176	0.184
C. defoliarti	0.194	0.192	0.188	0.201		0.187
C. crepuscularis	0.235	0.218	0.226	0.212	0.216	

	CAD-1	CAD-2	CAD-B	*C. (Selfia) sp.*	*C. crepuscularis*
CAD-1		0.047	0.061	0.221	0.245
CAD-2	0.049		0.055	0.231	0.249
CAD-B	0.064	0.057		0.228	0.239
C. (Selfia) sp.	0.266	0.280	0.275		0.232
C. crepuscularis	0.301	0.308	0.292	0.285	

	TPI-1	TPI-2	TPI-3	TPI-B	*C. (Selfia) sp.*	*C. butleri*
TPI-1		0.018	0.022	0.03	0.148	0.146
TPI-2	0.018		0.021	0.028	0.139	0.155
TPI-3	0.022	0.021		0.034	0.141	0.152
TPI-B	0.030	0.029	0.035		0.147	0.163
C. (Selfia) sp.	0.166	0.154	0.157	0.164		0.195
C. butleri	0.018	0.176	0.172	0.187	0.230	

Figure 1- Map of the United States representing the estimated ranges of *Culicoides sonorensis* (light grey), *C. variipennis* (dark grey), and *C. occidentalis* (diagonal stripes). *Culicoides* sampling locations are represented by the different open symbols. A circle represents individuals identified as *C. occidentalis*, a triangle represents individuals identified as *C. sonorensis*, a square represents individuals identified as *C. variipennis*, and a diamond represents individuals for which it was not possible to distinguish between *C. occidentalis* or *C. sonorensis* because only females were captured. If two different shapes are present at a single location, then both species were collected. See Appendix 1 for exact sampling locations and details about individual samples.

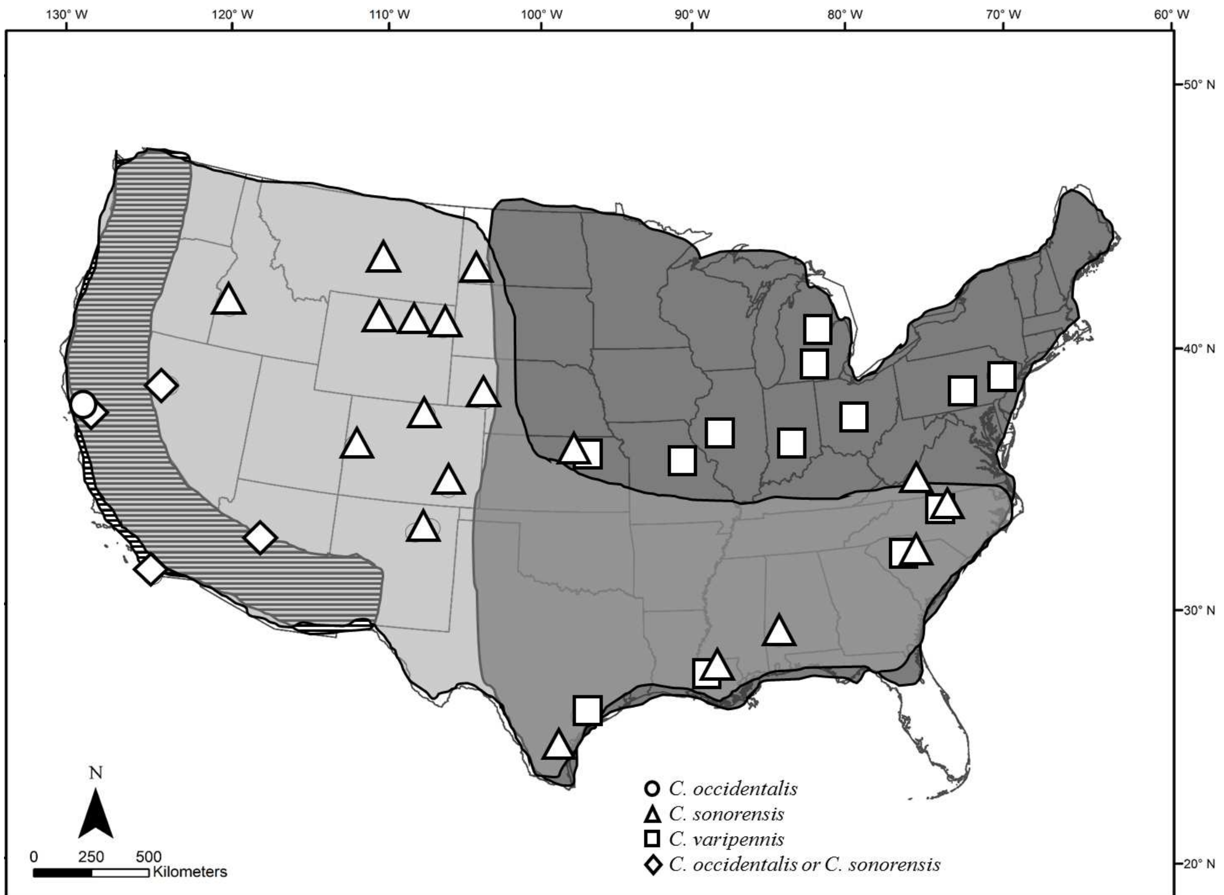


Figure 2-Bayesian gene trees reconstructed in MRBAYES for the two mtDNA loci, COI and COII, from *Culicoides* samples collected across the continental U.S. Included in the tree are the outgroup labels with species name and the *C. variipennis* complex samples represented by vertical bars and roman numerals. Asterisks (*) signify significant branch support in both the Bayesian (>75%) and maximum likelihood (>60%) algorithms. If the branch support was not statistically significant in both trees it was not noted.

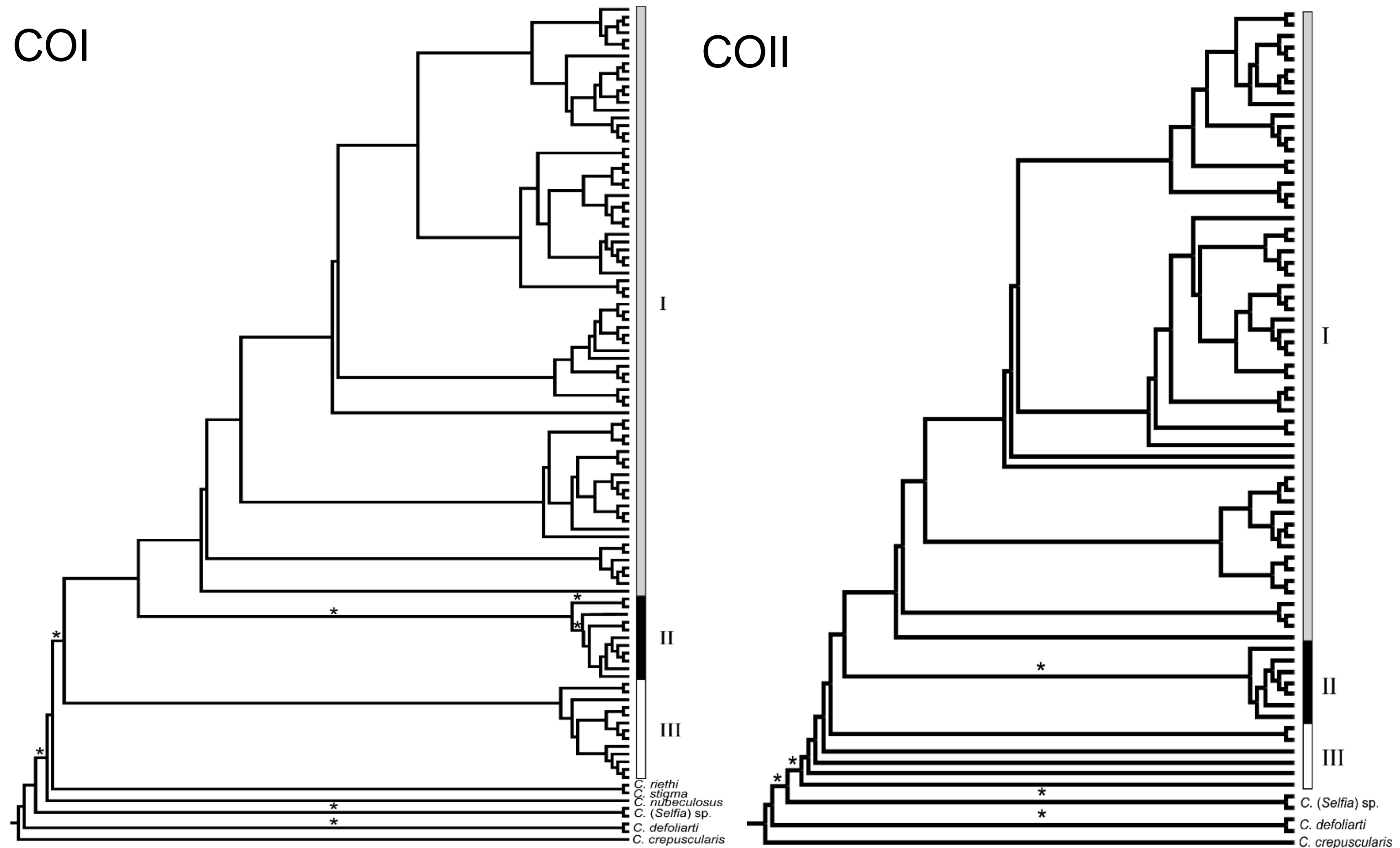


Figure 3-Bayesian gene trees reconstructed in MRBAYES for the two nuclear loci, CAD and TPI, from *Culicoides* samples collected across the continental U.S. Included in the tree are the outgroup labels with species name and the *C. variipennis* complex samples represented by vertical bars and roman numerals. Asterisks (*) signify significant branch support in both the Bayesian (>75%) and maximum likelihood (>60%) algorithms. If the branch support was not statistically significant in both trees it was not noted.

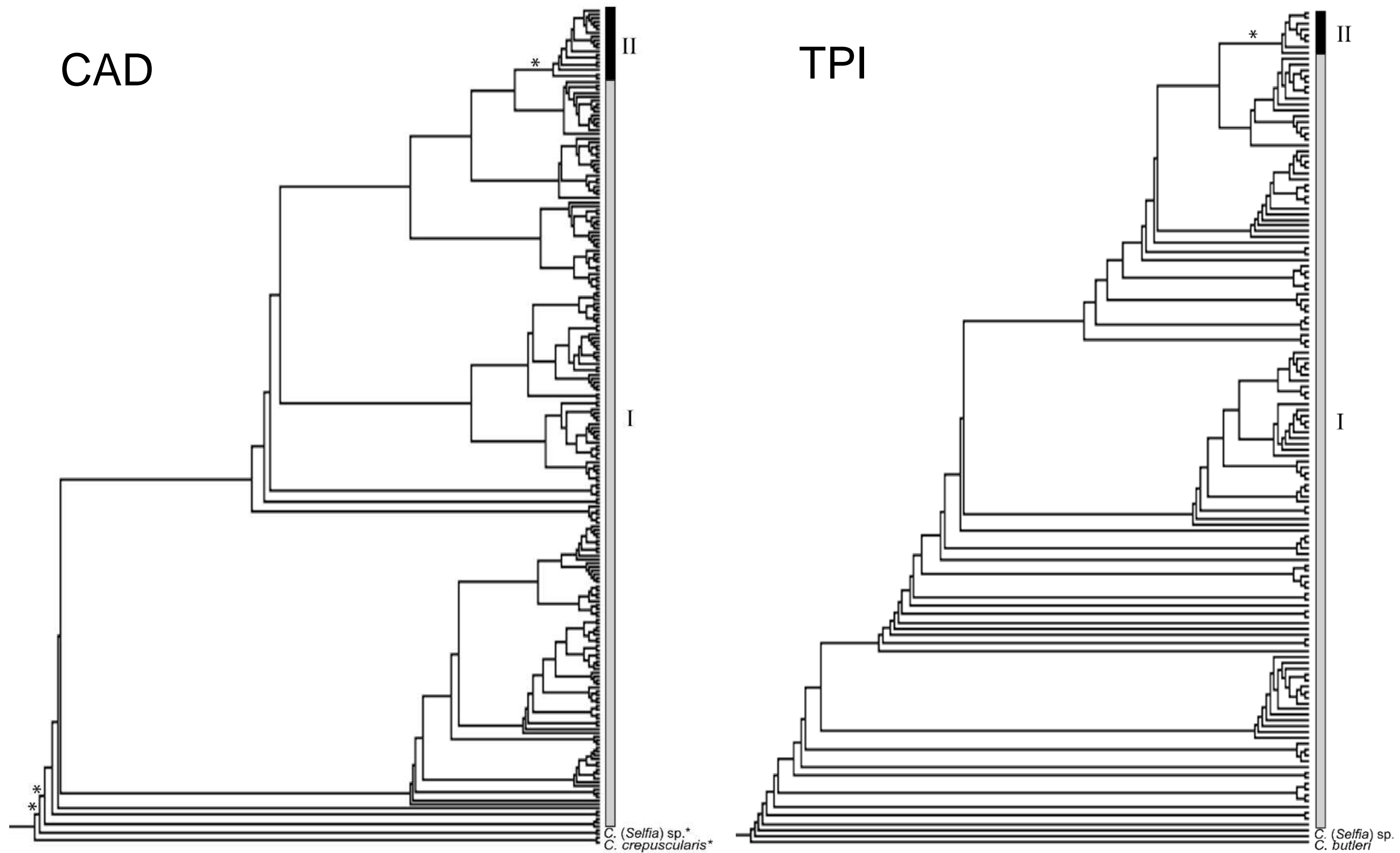


Figure 4- Mulit-locus Bayesian species tree estimated in *BEAST using two mitochondrial and two nuclear loci sequenced from *Culicoides* samples collected across the United States. The tips of the tree are labeled with the species identified using morphology. The branches are labeled with the posterior probability.

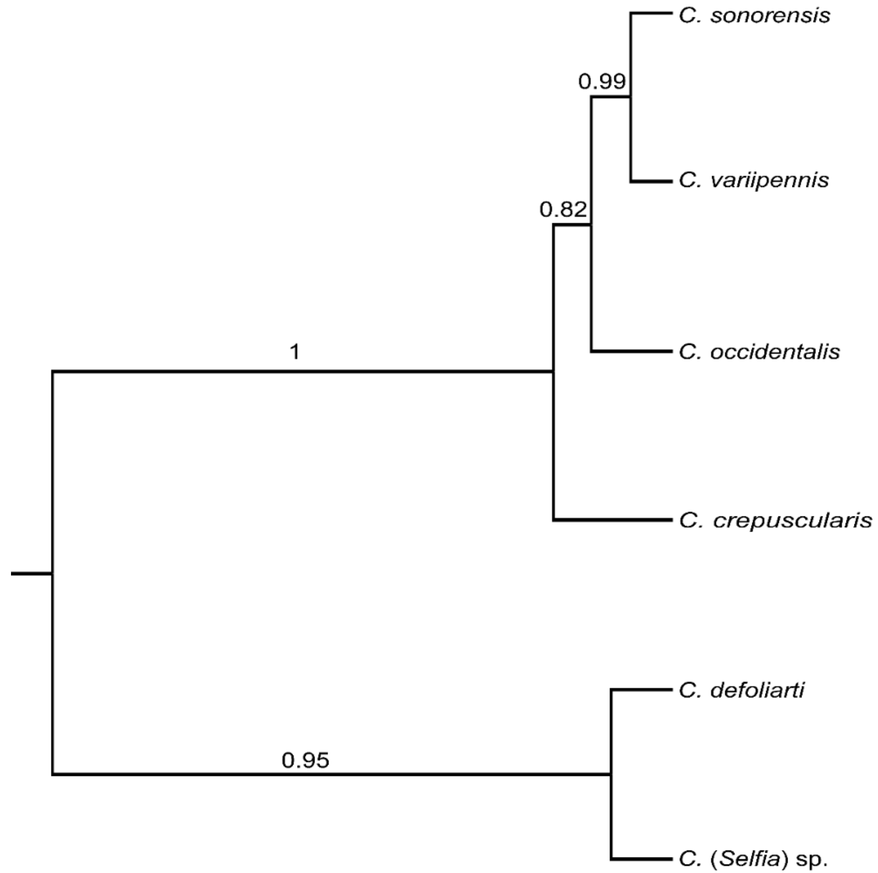


Figure 5-Median-joining haplotype networks constructed with POPART for two mtDNA loci, COI and COII, from *Culicoides variipennis* samples collected across the continental U.S. The nodes represent haplotypes and the sizes of the nodes are proportional to the number of *Culicoides* samples with that haplotype. The hatch marks on the edges represent the number of base pair differences between the haplotypes. The solid boxes represent haplogroups identified by more than 5 base pair changes. Haplogroup are designated using the abbreviation for the gene (e.g., COI) and capital letters indicate different haplogroups for the same gene.

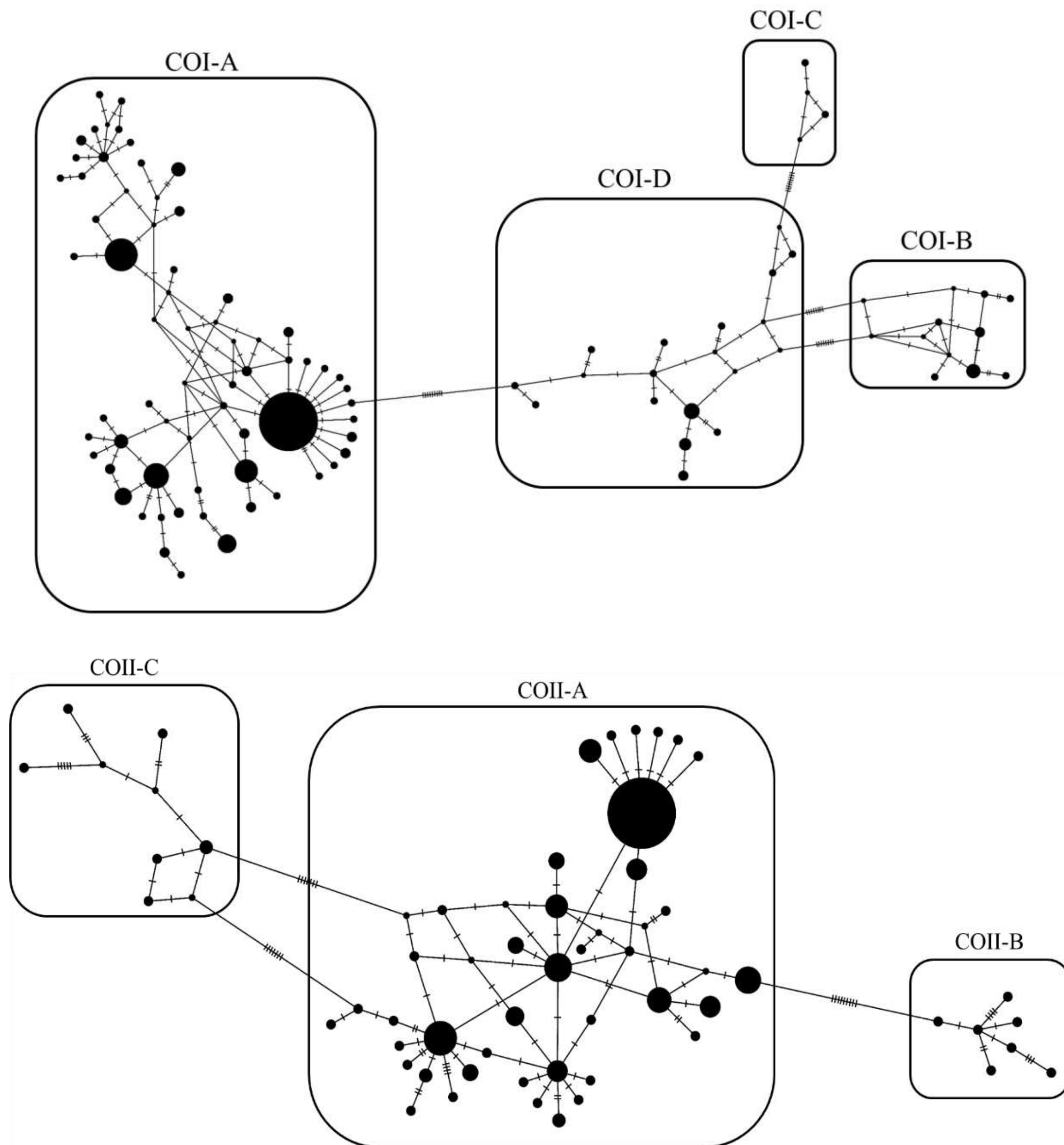


Figure 6-Median-joining haplotype networks constructed with POPART for two nuclear loci, CAD and TPI from *Culicoides variipennis* samples collected across the continental U.S. The nodes represent haplotypes and the sizes of the nodes are proportional to the number of *Culicoides* samples with that haplotype. The hatch marks on the edges represent the number of base pair differences between the haplotypes. The solid boxes represent haplogroups identified by more than 5 base pair changes. Haplogroup are designated using the abbreviation for the gene (e.g., CAD) and capital letters indicate different haplogroups for the same gene. The dashed lines represent genetic clusters identified by BAPS and are designated with the locus abbreviation and Arabic numerals.

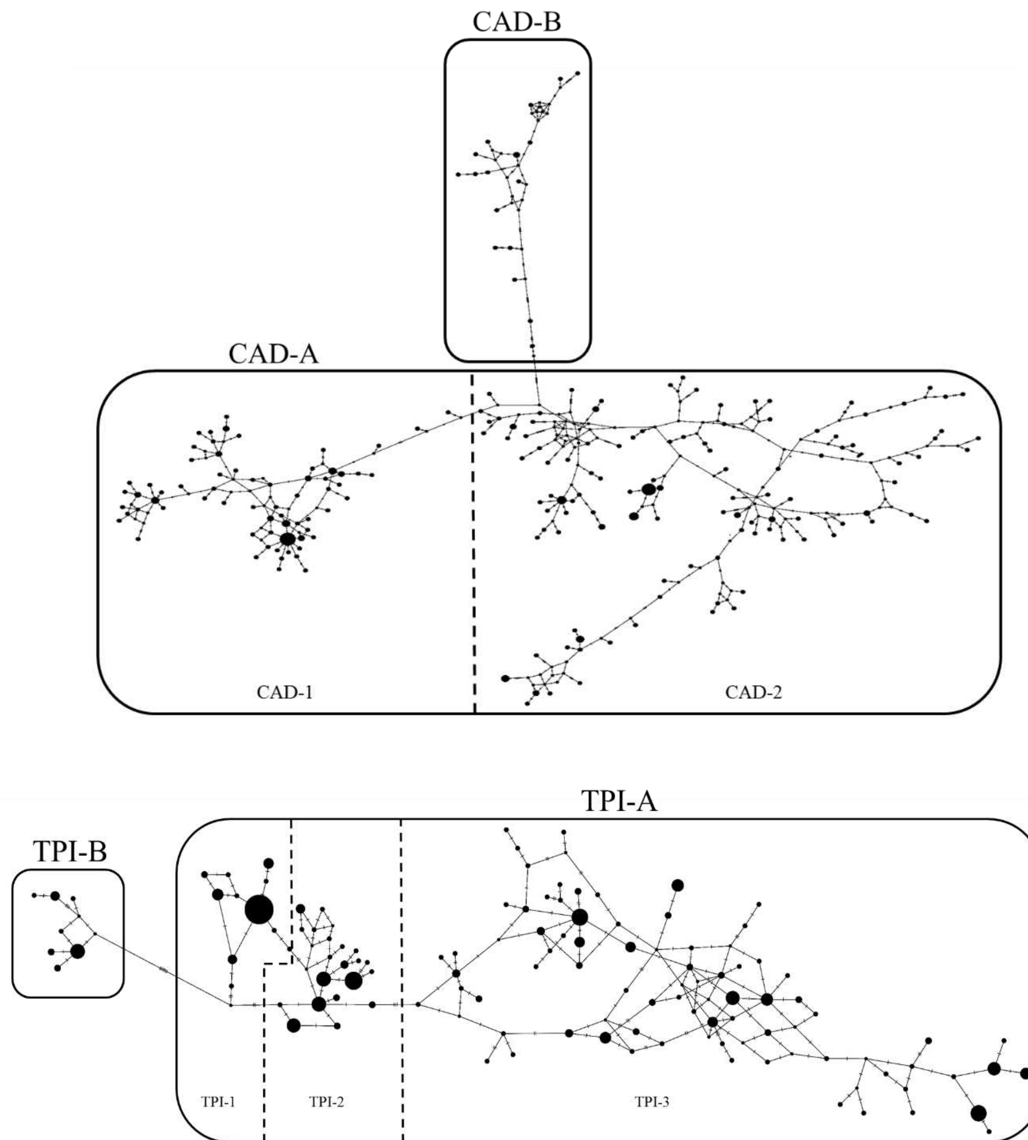


Figure 7-Plots of the mismatch distribution estimated in ARLEQUIN for each locus sequenced from *Culicoides variipennis* complex samples collected across the continental U.S. The x-axis is the distribution of the number of base pair differences and the y-axis is the total number of occurrences of the base pair changes. The bar plot is the observed distribution and the dashed line is the distribution of the expected base pair changes. All four loci are represented by their abbreviations: cytochrome oxidase (COI); cytochrome oxidase II (COII); carbamoyl phosphate synthetase region of the CAD (rudimentary) gene (CAD); and triose phosphate isomerase gene (TPI).

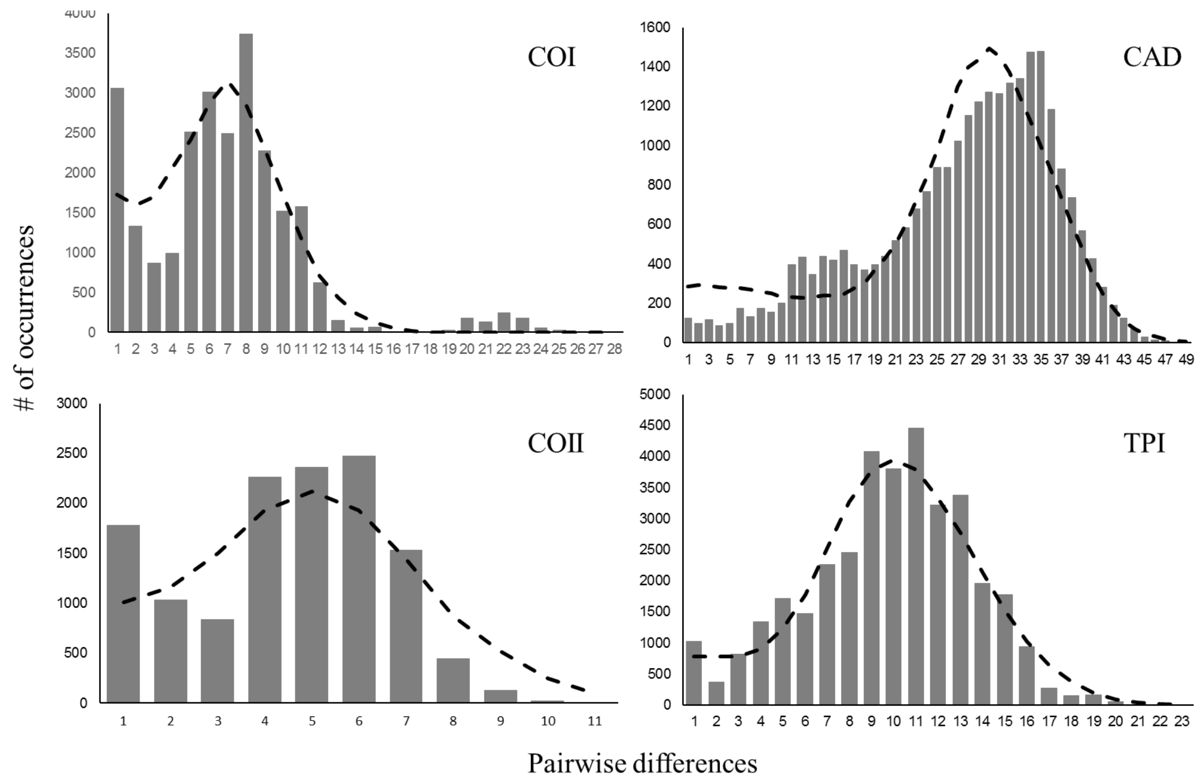
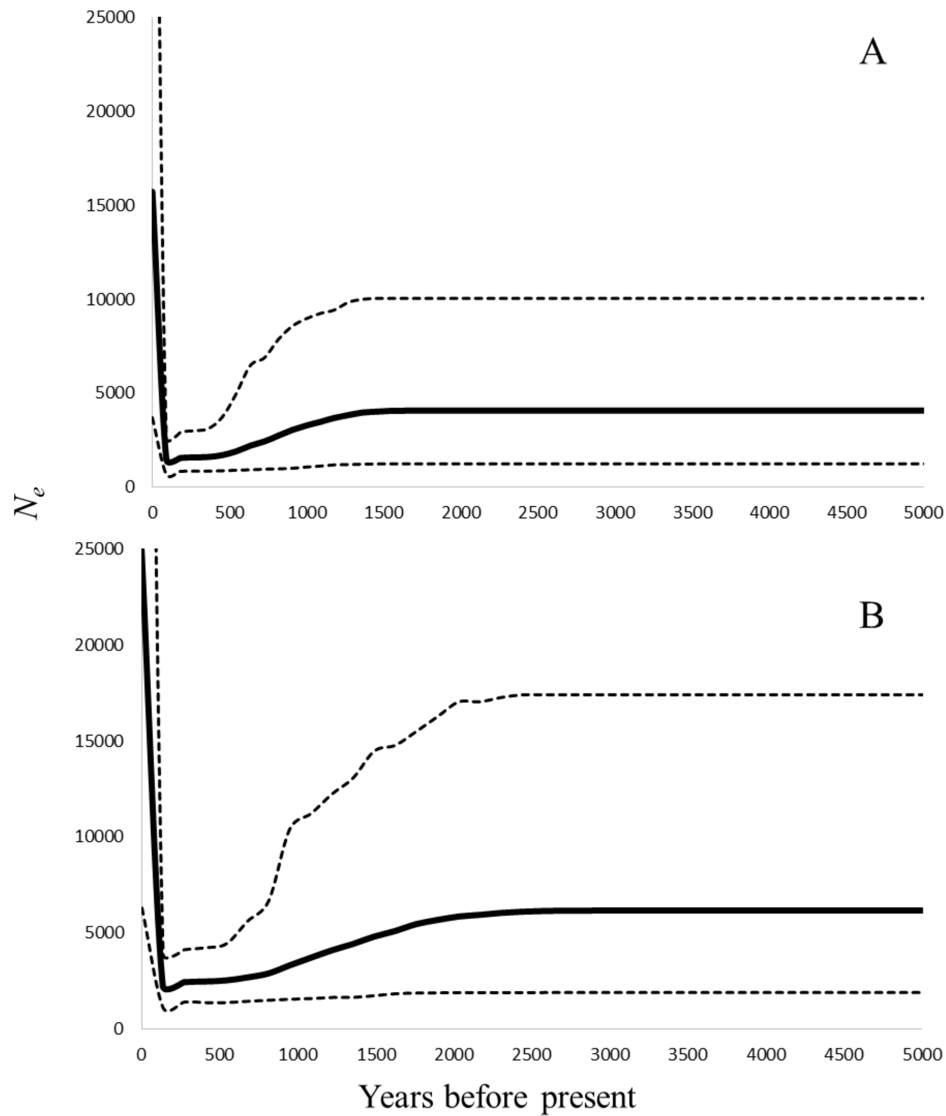


Figure 8-Bayesian skyline plot estimated in BEAST for the COI locus from the *Culicoides variipennis* complex collected across the continental U.S. The x-axis represents time in years and the y-axis is the estimated effective population size (N_e). The solid line is the mean estimate and the dashed lines are the upper and lower bounds. Plot A was generated using the molecular clock rate of 1.77% per million years and plot B was generated with the standard insect molecular clock of 1.17% per million years.



LITERATURE CITED

Allison, A.B., Goekjian, V.H., Potgieter, A.C., Wilson, W.C., Johnson, D.J., Mertens, P.P.C., Stallknecht, D.E., 2010. Detection of a novel reassortant epizootic hemorrhagic disease virus (EHDV) in the USA containing RNA segments derived from both exotic (EHDV-6) and endemic (EHDV-2) serotypes. Journal of General Virology 91, 430-439.

Ander, M., Troell, K., Chirico, J., 2013. Barcoding of biting midges in the genus *Culicoides*: a tool for species determination. Medical and Veterinary Entomology 27, 323-331.

Anderson, C.R., Spence, L., Downs, W.G., Aitken, T.H.G., 1961. Oropouche virus: a new human disease agent from Trinidad, West Indies. The American Journal of Tropical Medicine and Hygiene 10, 574-578.

Anderson, D., Frosch, B., Outlaw, J., 2007. Economic impact of the United States cervid farming industry. Agricultural and Food Policy Center, Texas A&M University, College Station, TX.

Atchley, W.R., 1967. The *Culicoides* of New Mexico.(Diptera: Ceratopogonidae). University of Kansas Publications.

Atchley, W.R., 1971. A comparative study of the cause and significance of morphological variation in adults and pupae of *Culicoides:* a factor analysis and multiple regression study. Evolution 25, 563-583.

Atkinson, C.T., Forrester, D.J., Greiner, E.C., 1988. Epizootiology of *Haemoproteus meleagridis* (Protozoa: Haemosporina) in Florida: seasonal transmission and vector abundance. Journal of Medical Entomology 25, 45-51.

Avise, J.C., Shapira, J.F., Daniel, S.W., Aquadro, C.F., Lansman, R.A., 1983. Mitochondrial DNA differentiation during the speciation process in *Peromyscus*. Molecular Biology and Evolution 1, 38-56.

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16, 37-48.

Bazin, E., Glémin, S., Galtier, N., 2006. Population size does not influence mitochondrial genetic diversity in animals. Science 312, 570-572.

Becker, M.E., Reeves, W.K., Dejean, S.K., Emery, M.P., Ostlund, E.N., Foil, L.D., 2010. Detection of bluetongue virus RNA in field-collected *Culicoides* spp. (Diptera: Ceratopogonidae) following the discovery of bluetongue virus serotype 1 in white-tailed deer and cattle in Louisiana. Journal of Medical Entomology 47, 269-273.

Bennett, G.F., 1961. On the specificity and transmission of some avian trypanosomes. Canadian Journal of Zoology 39, 17-33.

Bergold, G.H., Suárez, O.M., Munz, K., 1968. Multiplication in and transmission by *Aedes aegypti* of vesicular stomatitis virus. Journal of Invertebrate Pathology 11, 406-428.

Besansky, N.J., Severson, D.W., Ferdig, M.T., 2003. DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. Trends in Parasitology 19, 545-546.

Bishop, A.L., McKenzie, H.J., Barchia, I.M., Harris, A.M., 1996. Effect of temperature regimes on the development, survival and emergence of *Culicoides brevitarsis* Kieffer (Diptera: Ceratopogonidae) in bovine dung. Australian Journal of Entomology 35, 361-368.

Black, W.C., Moore, C.G., 2005. Population biology as a tool to study vector-borne diseases. In: Marquardt, W.C. (Ed.), Biology of Disease Vectors. Elsevier Academic Press, Burlington, MA, USA, pp. 187 - 206.

Blanton, F.S., Wirth, W.W., 1979. The sand flies (*Culicoides*) of Florida (Diptera: Ceratopogonidae). Florida Department of Agriculture and Consumer Services, Gainesville, Florida, USA.

Boorman, J., Mellor, P.S., Penn, M., Jennings, M., 1975. The growth of African horse-sickness virus in embryonated hen eggs and the transmission of virus by *Culicoides variipennis* Coquillett (Diptera, Ceratopogonidae). Archives of Virology 47, 343-349.

Borkent, A., 2005. The biting midges, the Ceratopogonidae (Diptera). In: Marquardt, W.C. (Ed.), Biology of Disease Vectors. Elsevier Academic Press, London, UK, pp. 113-126.

Borkent, A., 2015. World species of biting midges (Diptera: Ceratopogonidae). Royal British Columbia Museum, American Museum of Natural History, and Instituto Nacional de Biodiversidad, Salmon Arm, British Columbia, Canada.

Borkent, A., Grogan, W.L., 2009. Catalog of the New World biting midges north of Mexico (Diptera: Ceratopogonidae). Zootaxa 2273, 1-48.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST 2: A software platform for Bayesian evolutionary analysis. PLoS Comput Biol 10, e1003537.

Bram, R.A., George, J.E., Reichard, R.E., Tabachnick, W.J., 2002. Threat of foreign arthropod-borne pathogens to livestock in the United States. Journal of Medical Entomology 39, 405-416.

Brooks, D.R., Hoberg, E.P., 2006. Systematics and emerging infectious diseases: From management to solution. Journal of Parasitology 92, 426-429.

Brower, A.V., 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proceedings of the National Academy of Sciences 91, 6491-6495.

Buckley, J.J.C., 1934. On the development, in *Culicoides furens* Poey, of *Filaria* (= *Mansonella*) *ozzardi* Manson, 1897. Journal of Helminthology 12, 99-118.

Campbell, C.L., Vandyke, K.A., Letchworth, G.J., Drolet, B.S., Hanekamp, T., Wilson, W.C., 2005. Midgut and salivary gland transcriptomes of the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae). Insect Molecular Biology 14, 121-136.

Caracappa, S., Torina, A., Guercio, A., Vitale, F., Calabro, A., Purpari, G., Ferrantelli, V., Vitale, M., Mellor, P., 2003. Identification of a novel bluetongue virus vector species of *Culicoides* in Sicily. Veterinary Record 153, 71-74.

Carpenter, S., Groschup, M.H., Garros, C., Felippe-Bauer, M.L., Purse, B.V., 2013. *Culicoides* biting midges, arboviruses and public health in Europe. Antiviral Research 100, 102-113.

Carpenter, S., Szmaragd, C., Barber, J., Labuschagne, K., Gubbins, S., Mellor, P., 2008. An assessment of *Culicoides* surveillance techniques in northern Europe: have we underestimated a potential bluetongue virus vector? Journal of Applied Ecology 45, 1237-1245.

Carpenter, S., Wilson, A., Mellor, P.S., 2009. *Culicoides* and the emergence of bluetongue virus in northern Europe. Trends in Microbiology 17, 172-178.

Cheng, L., Connor, T.R., Sirén, J., Aanensen, D.M., Corander, J., 2013. Hierarchical and spatially explicit clustering of DNA sequences with BAPS software. Molecular Biology and Evolution 30, 1224-1228.

Collins, R.A., Cruickshank, R.H., 2013. The seven deadly sins of DNA barcoding. Molecular Ecology Resources 13, 969-975.

Corander, J., Sirén, J., Arjas, E., 2008. Bayesian spatial modeling of genetic population structure. Computational Statistics 23, 111-129.

Darpel, K.E., Batten, C.A., Veronesi, E., Shaw, A.E., Anthony, S., Bachanek-Bankowska, K., Kgosana, L., bin-Tarif, A., Carpenter, S., Muller-Doblies, U.U., Takamatsu, H.H., Mellor, P.S., Mertens, P.P., Oura, C.A., 2007. Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. Veterinary Record 161, 253-261.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287, 443-449.

Daszak, P., Tabor, G.M., Kilpatrick, A.M., Epstein, J.O.N., Plowright, R., 2004. Conservation medicine and a new agenda for emerging diseases. Annals of the New York Academy of Sciences 1026, 1-11.

De Liberato, C., Scavia, G., Lorenzetti, R., Scaramozzino, P., Amaddeo, D., Cardeti, G., Scicluna, M., Ferrari, G., Autorino, G.L., 2005. Identification of *Culicoides obsoletus* (Diptera: Ceratopogonidae) as a vector of bluetongue virus in central Italy. Veterinary Record 156, 301-304.

Dijkstra, E., van der Ven, I.J.K., Meiswinkel, R., Hölzel, D.R., van Rijn, P.A., Meiswinkel, R., 2008. *Culicoides chiopterus* as a potential vector of bluetongue virus in Europe. Veterinary Record 162, 422.

Downes, J.A., 1978. The *Culicoides variipennis* complex: a necessary re-alignment of nomenclature (Diptera: Ceratopogonidae). The Canadian Entomologist 110, 63-69.

Downing, J.A., 2014. Productivity of freshwater ecosystems and climate change. In: Freedman, B. (Ed.), Global Environmental Change. Springer Netherlands, Dordrecht, pp. 221-229.

Drolet, B.S., Campbell, C.L., Stuart, M.A., Wilson, W.C., 2005. Vector competence of *Culicoides sonorensis* (Diptera: Ceratopogonidae) for vesicular stomatitis virus. Journal of Medical Entomology 42, 409-418.

Drummond, A.J., Rambaut, A., Shapiro, B., Pybus, O.G., 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Molecular Biology and Evolution 22, 1185-1192.

Du Toit, R., 1944. The transmission of bluetongue and horsesickness by *Culicoides*. Onderstepoort Journal of Veterinary Science and Animal Industry 19, 7-16.

Ducheyne, E., De Deken, R., Bécu, S., Codina, B., Nomikou, K., Mangana-Vougiaki, O., Georgiev, G., Purse, B.V., Hendrickx, G., 2007. Quantifying the wind dispersal of *Culicoides* species in Greece and Bulgaria. 2007 1, 13.

Dujardin, J.P., Costa, J., Bustamante, D., Jaramillo, N., Catalá, S., 2009. Deciphering morphology in Triatominae: The evolutionary signals. Acta Tropica 110, 101-111.

Elbers, A., Koenraadt, C., Meiswinkel, R., 2015. Mosquitoes and *Culicoides* biting midges: vector range and the influence of climate change. Rev. Sci. Tech. Off. Int. Epiz 34, 123-137.

Enserink, M., 2008. Exotic disease of farm animals tests Europe's responses. Science 319, 710-711.

Ewald, P.W., 1995. The evolution of virulence: a unifying link between parasitology and ecology. J Parasitol 81, 659-669.

Excoffier, L., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10, 564-567.

Falconi, C., López-Olvera, J.R., Gortázar, C., 2011. BTV infection in wild ruminants, with emphasis on red deer: A review. Veterinary Microbiology 151, 209-219.

Fallis, A.M., Bennett, G.F., 1960. Description of *Haemoproteus canachites* N. sp. (Sporozoa:Haemopoteidae) and sporogony in *Culicoides* (Dipter:Ceratopogonidae). Canadian Journal of Zoology 38, 455-464.

Fallis, A.M., Bennett, G.F., 1961a. Ceratopogonidae as intermediate hosts for *Haemoproteus* and other parasites. Mosquito News 21, 21-28.

Fallis, A.M., Bennett, G.F., 1961b. Sporogony of *Leucocytozoon* and *Haemoproteus* in sumuliids and ceratopogonids and a reclassification of the Haemosporidiida. Canadian Journal of Zoology 39, 215-228.

Fallis, A.M., Wood, D.M., 1957. Biting midges (Diptera: Ceratopogonidae) as intermediate hosts for *Haemoproteus* of ducks Canadian Journal of Zoology 35, 425-435.

Felsenstein, J., 1993. PHYLIP: phylogenetic inference package, version 3.5c. Department of Genetics, University of Washington.

Foil, L.D., Klei, T.R., Miller, R.I., Church, G.E., Foil, C.S., French, D.D., Smith, J.N., 1987. Seasonal changes in density and tissue distribution of *Onchocerca cervicalis*

Microfilariae in ponies and related changes in *Culicoides variipennis* populations in Louisiana. The Journal of Parasitology 73, 320-326.

Foster, N., Breckon, R., Luedke, A., Jones, R., 1977. Transmission of two strains of epizootic hemorrhagic disease virus in deer by *Culicoides variipennis*. Journal of Wildlife Diseases 13, 9-16.

Foster, N., Jones, R., McCrory, B., 1963. Preliminary investigations on insect transmission of bluetongue virus in sheep. American Journal of Veterinary Research 24, 1195.

Fu, Y.-X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915-925.

García-Bocanegra, I., Arenas-Montes, A., Lorca-Oró, C., Pujols, J., González, M.A., Napp, S., Gómez-Guillamón, F., Zorrilla, I., Miguel, E.S., Arenas, A., 2011. Role of wild ruminants in the epidemiology of bluetongue virus serotypes 1, 4 and 8 in Spain. Veterinary Research 42, 88.

Garrett-Jones, C., 1964. Prognosis for interruption of malaria transmission through assessment of the mosquito's vectorial capacity. Nature 204, 1173-1175.

Gaydos, J.K., Crum, J.M., Davidson, W.R., Cross, S.S., Owen, S.F., Stallknecht, D.E., 2004. Epizootiology of an epizootic hemorrhagic disease outbreak in West Virginia. Journal of Wildlife Diseases 40, 383-393.

Gibbs, E.P.J., Tabachnick, W.J., Holt, T.J., Stallknecht, D.E., 2008. U.S. concerns over bluetongue. Science 320, 872.

Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., Posada, D., 2010. ALTER: program-oriented conversion of DNA and protein alignments. Nucleic Acids Research 38 (suppl 2), W14-W18.

Gould, A.R., Hyatt, A.D., 1994. The orbivirus genus. Diversity, structure, replication and phylogenetic relationships. Comparative Immunology, Microbiology and Infectious Diseases 17, 163-188.

Grado, S.C., Hunt, K.M., Whiteside, M.W., 2007. Economic impacts of white-tailed deer hunting in Mississippi. Proceedings of the Southeastern Association of Fish and Wildlife Agencies 61, 59-67.

Greiner, E., Barber, T., Pearson, J., Kramer, W., Gibbs, E., 1984a. Orbiviruses from *Culicoides* in Florida. Progress in clinical and biological research 178, 195-200.

Greiner, E.C., Garris, G.I., Rollo, R.T., Knausenberger, W.I., Jones, J.E., Gibbs, E.P.J., 1984b. Preliminary studies on the *Culicoides* spp. as potential vectors of bluetongue in the Caribbean region. Preventive Veterinary Medicine 2, 389-399.

Grogan Jr., W.L., Phillips, R.A., 2008. A new species of biting midge in the subgenus *Monoculicoides* of *Culicoides* from Utah (Diptera: Ceratopogonidae). Proceedings of the Entomological Society of Washington 110, 196-203.

Harbach, R.E., 2004. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bulletin of Entomological Research 94, 537-553.

Hardy, J.L., Scrivani, R.P., Lyness, R.N., Nelson, R.L., Boberts, D.R., 1970. Ecologic studies on buttonwillow virus in Kern County, California, 1961–1968. The American Journal of Tropical Medicine and Hygiene 19, 552-563.

Harrup, L.E., Bellis, G.A., Balenghien, T., Garros, C., 2015. *Culicoides* Latreille (Diptera: Ceratopogonidae) taxonomy: Current challenges and future directions. Infection, Genetics and Evolution 30, 249-266.

Harrus, S., Baneth, G., 2005. Drivers for the emergence and re-emergence of vector-borne protozoal and bacterial diseases. International Journal for Parasitology 35, 1309-1318.

Hedges, S.B., Sibley, C.G., 1994. Molecules vs. morphology in avian evolution: the case of the "pelecaniform" birds. Proceedings of the National Academy of Sciences 91, 9861-9865.

Heled, J., Drummond, A.J., 2010. Bayesian inference of species trees from multilocus data. Molecular Biology and Evolution 27, 570-580.

Hellgren, O., Pérez-Tris, J., Bensch, S., 2009. A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. Ecology 90, 2840-2849.

Hensleigh, D.A., Atchley, W.R., 1977. Morphometric variability in natural and laboratory populations of *Culicoides variipennis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 14, 379-386.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J.H., 2008. How many species are infected with Wolbachia? – a statistical analysis of current data. FEMS Microbiology Letters 281, 215-220.

Hoar, B.R., Carpenter, T.E., Singer, R.S., Gardner, I.A., 2003. Regional risk of exporting cattle seropositive for bluetongue virus from the United States. American Journal of Veterinary Research 64, 520-529.

Hoffmann, B., Bauer, B., Bauer, C., Bätza, H.-J., Beer, M., Clausen, P.-H., Geier, M., Gethmann, J.M., Kiel, E., Liebisch, G., Liebisch, A., Mehlhorn, H., Schaub, G.A., Werner, D., Conraths, F.J., 2009. Monitoring of putative vectors of bluetongue virus serotype 8, Germany. Emerging Infectious Diseases 15, 1481-1484.

Holbrook, F.R., Tabachnick, W.J., 1995. *Culicoides variipennis* (Diptera: Ceratopogonidae) complex in California. Journal of Medical Entomology 32, 413-419.

Holbrook, F.R., Tabachnick, W.J., Brady, R., 1996. Genetic variation in populations of *Culicoides variipennis* complex in the six New England states, U.S.A. Medical and Veterinary Entomology 10, 173-180.

Holbrook, F.R., Tabachnick, W.J., Schmidtmann, E.T., McKinnon, C.N., Bobian, R.J., Grogan Jr, W.L., 2000. Sympatry in the *Culicoides variipennis* complex (Diptera: Ceratopogonidae): a taxonomic reassessment. Journal of Medical Entomology 37, 65-76.

Hoogendam, K., 2007. International study on the economic consequences of outbreaks of bluetongue serotype 8 in north-western Europe. Van Hall Institute, Leeuwarden, The Netherlands.

Hourrigan, J.L., Klingsporn, A.L., 1975. Epizootiology of bluetongue: the situation in the United States of America. Australian Veterinary Journal 51, 203-208.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Huerta, H., Rodríguez Castrejón, A.M., Grogan Jr, W.L., Ibánez-Bernal, S., 2012. New records of biting midges of the genus *Culicoides* Latreille from Mexico (Diptera: Ceratopogonidae). Insecta Mundi 0211, 1-20.

Hurst, G.D.D., Jiggins, F.M., 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proceedings of the Royal Society of London B: Biological Sciences 272, 1525-1534.

Jackson, J.A., Tinsley, R.C., 2005. Geographic and within-population structure in variable resistance to parasite species and strains in a vertebrate host. International Journal for Parasitology 35, 29-37.

Jamnback, H., Wirth, W., 1963. The species of *Culicoides* related to *obsoletus* in eastern North America (Diptera: Ceratopogonidae). Annals of the Entomological Society of America 56, 185-198.

Jenkins, E.J., Simon, A., Bachand, N., Stephen, C., 2015. Wildlife parasites in a One Health world. Trends in Parasitology 31, 174-180.

Jennings, M., Mellor, P.S., 1989. *Culicoides*: Biological vectors of akabane virus. Veterinary Microbiology 21, 125-131.

Johnson, D.J., Ostlund, E.N., Stallknecht, D.E., Goekjian, V.H., Jenkins-Moore, M., Harris, S.C., 2006. First report of bluetongue virus serotype 1 isolated from a white-tailed deer in the United States. Journal of Veterinary Diagnostic Investigation 18, 398-401.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature 451, 990-993.

Jones, R., 1965. Epidemiological notes: incidence of *Culicoides variipennis* in an outbreak of bluetongue disease. Mosquito News 25, 217-218.

Jones, R., Schmidtmann, E., Foster, N., 1983. Vector-competence studies for bluetongue and epizootic hemorrhagic disease viruses with *Culicoides venustus* (Ceratopogonidae). Mosquito News 43.

Jones, R.H., Roughton, R.D., Foster, N.M., Bando, B.M., 1977. *Culicoides*, the vector of epizootic hemorrhagic disease deer in Kentucky in 1971. Journal of Wildlife Diseases 13, 2-8.

Kamali, M., Xia, A., Tu, Z., Sharakhov, I.V., 2012. A new chromosomal phylogeny supports the repeated origin of vectorial capacity in malaria mosquitoes of the *Anopheles gambiae* complex. PLoS Pathogens 8, e1002960.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33, 511-518.

Katoh, K., Misawa, K., Kuma, K.i., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30, 3059-3066.

Kedmi, M., Van Straten, M., Ezra, E., Galon, N., Klement, E., 2010. Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds. Journal of Dairy Science 93, 2486-2495.

Khalaf, K., 1954. The speciation of the genus *Culicoides* (Diptera, Heleidae). Annals of the Entomological Society of America 47, 34-51.

Khan, R.A., Fallis, A.M., 1971. A note on the sporogony of *Parahaemoproteus velans* (= *Haemoproteus velans* Coatney and Roudabush) (Haemosporidia: Haemoproteidae) in species of *Culicoides*. Canadian Journal of Zoology 49, 420-421.

Kilpatrick, A.M., 2011. Globalization, land use, and the invasion of West Nile virus. Science 334, 323-327.

Kilpatrick, A.M., Fonseca, D.M., Ebel, G.D., Reddy, M.R., Kramer, L.D., 2010. Spatial and temporal variation in vector competence of *Culex pipiens* and *Cx. restuans* mosquitoes for West Nile virus. The American Journal of Tropical Medicine and Hygiene 83, 607-613.

Kitchen, A., Shackelton, L.A., Holmes, E.C., 2011. Family level phylogenies reveal modes of macroevolution in RNA viruses. Proceedings of the National Academy of Sciences 108, 238-243.

Knowles, S.C.L., Palinauskas, V., Sheldon, B.C., 2010. Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. Journal of Evolutionary Biology 23, 557-569.

Koenraadt, C., Balenghien, T., Carpenter, S., Ducheyne, E., Elbers, A., Fife, M., Garros, C., Ibanez-Justicia, A., Kampen, H., Kormelink, R., Losson, B., van der Poel, W., De Regge, N., van Rijn, P., Sanders, C., Schaffner, F., Sloet van Oldruitenborgh-Oosterbaan, M., Takken, W., Werner, D., Seelig, F., 2014. Bluetongue, Schmallenberg - what is next? *Culicoides*-borne viral diseases in the 21st Century. BMC veterinary research 10, 77.

Kramer, W.L., Greiner, E.C., Gibbs, E.P.J., 1985a. Seasonal variations in populations size, fecundity, and parity rates of *Culicoides insignis* (Diptera: Ceratopogonidae) in Florida, USA. Journal of Medical Entomology 22, 163-169.

Kramer, W.L., Greiner, E.C., Gibbs, E.P.J., 1985b. A survey of *Culicoides* midges (Diptera: Ceratopogonidae) associated with cattle operations in Florida, USA. Journal of Medical Entomology 22, 153-162.

Kramer, W.L., Jones, R.H., Holbrook, F.R., Walton, T.E., Calisher, C.H., 1990. Isolation of arboviruses from *Culicoides* midges (Diptera: Ceratopogonidae) in Colorado during an epizootic of vesicular stomatitis New Jersey. Journal of Medical Entomology 27, 487-493.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution accepted.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the Northeastern United States. Science 286, 2333-2337.

Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S., 2012. PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. Molecular Biology and Evolution 29, 1695-1701.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

Lau, S.K.P., Woo, P.C.Y., Li, K.S.M., Huang, Y., Tsoi, H.-W., Wong, B.H.L., Wong, S.S.Y., Leung, S.-Y., Chan, K.-H., Yuen, K.-Y., 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proceedings of the National Academy of Sciences of the United States of America 102, 14040-14045.

Leather, S.R., 2009. Taxonomic chauvinism threatens the future of entomology. Biologist 56, 10.

Lemmon, A.R., Brown, J.M., Stanger-Hall, K., Lemmon, E.M., 2009. The Effect of ambiguous data on phylogenetic estimates obtained by maximum likelihood and Bayesian inference. Systematic Biology 58, 130-145.

Levins, R., 1965. Theory of fitness in a heterogeneous environment. V. Optimal Genetic Systems. Genetics 52, 891-904.

Levins, R., 1969. Thermal acclimation and heat resistance in *Drosophila* species. The American Naturalist 103, 483-499.

Linden, A., Desmecht, D., Volpe, R., Wirtgen, M., Gregoire, F., Pirson, J., Paternostre, J., Kleijnen, D., Schirrmeier, H., Beer, M., Garigliany, M.-M., 2012. Epizootic spread of Schmallenberg virus among wild cervids, Belgium, Fall 2011. Emerging Infectious Diseases 18, 2006-2008.

Linley, J.R., 1985. Biting midges (Diptera: Ceratopogonidae) as vectors of nonviral animal pathogens. Journal of Medical Entomology 22, 589-599.

Maclachlan, N., Drew, C., Darpel, K., Worwa, G., 2009. The pathology and pathogenesis of bluetongue. Journal of comparative pathology 141, 1-16.

MacLachlan, N.J., Guthrie, A.J., 2010. Re-emergence of bluetongue, African horse sickness, and other *Orbivirus* diseases. Vet. Res. 41, 35.

Maclachlan, N.J., Mayo, C.E., 2013. Potential strategies for control of bluetongue, a globally emerging, *Culicoides*-transmitted viral disease of ruminant livestock and wildlife. Antiviral Research 99, 79-90.

Martinsen, E.S., Perkins, S.L., Schall, J.J., 2008. A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-history traits and host switches. Molecular Phylogenetics and Evolution 47, 261-273.

Marzal, A., Lope, F., Navarro, C., Møller, A., 2005. Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia 142, 541-545.

Mead, D.G., Ramberg, F.B., Besselsen, D.G., Maré, C.J., 2000. Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice. Science 287, 485-487.

Mecham, J.O., Stallknecht, D., Wilson, W.C., 2003. The S7 gene and VP7 protein are highly conserved among temporally and geographically distinct American isolates of epizootic hemorrhagic disease virus. Virus research 94, 129-133.

Meinswinkel, R., Nevill, E.M., Venter, G.J., 1994. Vectors: *Culicoides* spp. In: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. (Eds.), Infectious disease of livestock with special reference to Southern Africa. Oxford University Press, Cape Town, pp. 68-89.

Meiswinkel, R., Van Rijn, P., Leijs, P., Goffredo, M., 2007. Potential new *Culicoides* vector of bluetongue virus in northern Europe. Veterinary Record 161, 564-565.

Mellor, P.S., 1975. Studies on *Onchocerca cervicalis* Railliet and Henry 1910: V. The development of *Onchocerca cervicalis* larvae in the vectors. Journal of Helminthology 49, 33-42.

Mellor, P.S., Boorman, J., Baylis, M., 2000. *Culicoides* biting midges: their role as arbovirus vectors. Annual Review of Entomology 45, 307-340.

Mellor, P.S., Wittmann, E.J., 2002. Bluetongue virus in the Mediterranean Basin 1998-2001. Veterinary Journal 164, 20-37.

Miller, M.A., Pfeiffer, W., Schwartz, T., 2010a. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop (GCE), 2010. IEEE, pp. 1-8.

Miller, M.M., Brown, J., Cornish, T., Johnson, G., Mecham, J.O., Reeves, W.K., Wilson, W., 2010b. Investigation of a bluetongue disease epizootic caused by bluetongue virus serotype 17 in sheep in Wyoming. Journal of the American Veterinary Medical Association 237, 955-959.

Mo, C.L., Thompson, L.H., Homan, E.J., Oviedo, M.T., Greiner, E.C., González, J., Sáenz, M.R., 1994. Bluetongue virus isolations from vectors and ruminants in Central America and the Caribbean. American Journal of Veterinary Research 55, 211-215.

Moczek, A.P., 2010. Phenotypic plasticity and diversity in insects. Philosophical Transactions of the Royal Society of London B: Biological Sciences 365, 593-603.

Montana Fish, W., and Parks, 2011. Press release: EHD takes toll on Eastern Montana white-tailed deer.

Mullen, G., Anderson, R., 1998. Transmission of orbiviruses by *Culicoides* Latreille species (Ceratopogonidae) among cattle and white-tailed deer in the southeastern United States. Abstracts Volume. Fourth International Congress of Dipterology, pp. 155-156.

Mullen, G., Jones, R., Braverman, Y., Nusbaum, K., 1985a. Laboratory infections of *Culicoides debilipalpis* and *C. stellifer* (Diptera: Ceratopogonidae) with bluetongue virus. Progress in Clinical and Biological Research 178, 239.

Mullen, G.R., 2009. Biting Midges (Ceratopogonidae). In: Mullen, G.R., Durden, L.A. (Eds.), Medical and Veterinary Entomology. Academic Press, Burlington, MA, pp. 169-188.

Mullen, G.R., Hayes, M.E., Nusbaum, K.E., 1985b. Potential vectors of bluetongue and epizootic hemorrhagic disease viruses of cattle and white-tailed deer in Alabama. Progress in Clinical and Biological Research 178, 201-206.

Mullens, B.A., Cardona, C.J., McClellan, L., Szijj, C.E., Owen, J.P., 2006. *Culicoides bottimeri* as a vector of *Haemoproteus lophortyx* to quail in California, USA. Veterinary Parasitology 140, 35-43.

Mullens, B.A., Dada, C.E., 1992a. Insects feeding on desert bighorn sheep, domestic rabbits, and japanese quail in the Santa Rosa Mountains of Southern California. Journal of Wildlife Diseases 28, 476-480.

Mullens, B.A., Dada, C.E., 1992b. Spatial and seasonal distribution of potential vectors of hemorrhagic disease viruses to peninsular bighorn sheep in the Santa Rosa mountains of southern California. Journal of Wildlife Diseases 28, 192-205.

Naugle, D.E., Aldridge, C.L., Walker, B.L., Cornish, T.E., Moynahan, B.J., Holloran, M.J., Brown, K., Johnson, G.D., Schmidtmann, E.T., Mayer, R.T., Kato, C.Y., Matchett, M.R., Christiansen, T.J., Cook, W.E., Creekmore, T., Falise, R.D., Thomas Rinkes, E., Boyce, M.S., 2004. West Nile virus: pending crisis for greater sage-grouse. Ecology Letters 7, 704-713.

Nayduch, D., Cohnstaedt, L.W., Saski, C., Lawson, D., Kersey, P., Fife, M., Carpenter, S., 2014. Studying *Culicoides* vectors of BTV in the post-genomic era: Resources, bottlenecks to progress and future directions. Virus Research 182, 43-49.

Nettles, V., Davidson, W., Stallknecht, D., 1992. Surveillance for hemorrhagic disease in white-tailed deer and other wild ruminants, 1980–1989. Proceedings of the annual conference of the Southeastern Association of Fish and Wildlife Agencies, pp. 138-146.

Noon, T.H., Wesche, S.L., Cagle, D., Mead, D.G., Bicknell, E.J., Bradley, G.A., Riplog-Peterson, S., Edsall, D., Reggiardo, C., 2002. Hemorrhagic disease in bighorn sheep in Arizona. Journal of wildlife diseases 38, 172-176.

Osborne, C.J., Mayo, C.E., Mullens, B.A., McDermott, E.G., Gerry, A.C., Reisen, W.K., MacLachlan, N.J., 2015. Lack of evidence for laboratory and natural vertical transmission of bluetongue virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae).

Ostlund, E., Moser, K., Johnson, D., Pearson, J., Schmitt, B., 2004. Distribution of bluetongue in the United States of America, 1991–2002. Veterinaria Italiana 40, 83-88.

Packer, L., Grixti, J.C., Roughley, R.E., Hanner, R., 2009. The status of taxonomy in Canada and the impact of DNA barcoding. Canadian Journal of Zoology 87, 1097-1110.

Pagès, N., Muñoz-Muñoz, F., Talavera, S., Sarto, V., Lorca, C., Núñez, J.I., 2009. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. Veterinary Parasitology 165, 298-310.

Papadopoulou, A., Anastasiou, I., Vogler, A.P., 2010. Revisiting the insect mitochondrial molecular clock: The mid-Aegean trench calibration. Molecular Biology and Evolution 27, 1659-1672.

Patterson, C., Williams, D.M., Humpries, C.J., 1993. Congruence between molecular and morphological phylogenies. Annual Review of Ecology and Systematics 24, 153-188.

Pérez de León, A.A., O'Toole, D., Tabachnick, W.J., 2006. Infection of Guinea pigs with vesicular stomatitis New Jersey virus transmitted by *Culicoides sonorensis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 43, 568-573.

Pérez de León, A.A., Tabachnick, W.J., 2006. Transmission of vesicular stomatitis New Jersey virus to cattle by the biting midge *Culicoides sonorensis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 43, 323-329.

Petersen, L.R., Hayes, E.B., 2008. West Nile virus in the Americas. Medical Clinics of North America 92, 1307-1322.

Pinheiro, F., Travassos da Rosa, A., Gomes, M., LeDuc, J., Hoch, A., 1982. Transmission of Oropouche virus from man to hamster by the midge *Culicoides paraensis*. Science 215, 1251-1253.

Pinheiro, F.P., Hoch, A.L., Gomes, M.L., Roberts, D.R., 1981a. Oropouche virus. IV. Laboratory transmission by *Culicoides paraensis*. The American Journal of Tropical Medicine and Hygiene 30, 172-176.

Pinheiro, F.P., Travassos da Rosa, A., Travassos da Rosa, J., Ishak, R., Gomes, M., Ouva, O., LeDuc, J., 1981b. Oropouche Virus: A review of clinical, epidemiological and ecological findings. American Journal of Tropical Medicine and Hygiene 30, 149-160.

Posada, D., Crandall, K.A., 2001. Intraspecific gene genealogies: trees grafting into networks. Trends in Ecology & Evolution 16, 37-45.

ProMED-mail, 2012. Epizootic hemorrhagic disease, cervids - USA (23): (NE)

Purse, B.V., Carpenter, S., Venter, G.J., Bellis, G., Mullens, B.A., 2015. Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of *Culicoides*-borne viruses. Annual Review of Entomology 60, 373-392.

Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P.C., Baylis, M., 2005. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Micro 3, 171-181.

Rambaut, A., 2014. FigTree version 1.4. http://tree.bio.ed.ac.uk/software/figtree/

Rogers, A.R., Harpending, H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. Molecular Biology and Evolution 9, 552-569.

Rooney, T.P., Waller, D.M., 2003. Direct and indirect effects of white-tailed deer in forest ecosystems. Forest Ecology and Management 181, 165-176.

Rosenstock, S., Ramberg, F., Collins, J., Rabe, M., 2003. *Culicoides mohave* (Diptera: Ceratopogonidae): New occurrence records and potential role in transmission of hemorrhagic disease. Journal of Medical Entomology 40, 577-579.

Ruder, M.G., Lysyk, T.J., Stallknecht, D.E., Foil, L.D., Johnson, D.J., Chase, C.C., Dargatz, D.A., Gibbs, E.P.J., 2015. Transmission and epidemiology of bluetongue and epizootic hemorrhagic disease in North America: Current perspectives, research gaps, and future directions. Vector-Borne and Zoonotic Diseases 15, 348-363.

Ruiz-Fons, F., Sánchez-Matamoros, A., Gortázar, C., Sánchez-Vizcaíno, J.M., 2014. The role of wildlife in bluetongue virus maintenance in Europe: Lessons learned after the natural infection in Spain. Virus Research 182, 50-58.

Sabio, I.J., Mackay, A.J., Roy, A., Foil, L.D., 2006. Detection of West Nile virus RNA in pools of three species of Ceratopogonids (Diptera: Ceratopogonidae) collected in Louisiana. Journal of Medical Entomology 43, 1020-1022.

Samal, S.K., El-Hussein, A., Holbrook, F.R., Beaty, B.J., Ramig, R.F., 1987. Mixed infection of *Culicoides variipennis* with bluetongue virus serotypes 10 and 17: evidence for high frequency reassortment in the vector. Journal of General Virology 68, 2319-2329.

Savini, G., Afonso, A., Mellor, P., Aradaib, I., Yadin, H., Sanaa, M., Wilson, W., Monaco, F., Domingo, M., 2011. Epizootic heamorragic disease. Research in Veterinary Science 91, 1-17.

Schmidtmann, E.T., 2006. Testing the relationship between dissolved salts in aquatic habitats and immature populations of the *Culicoides variipennis* Complex (Diptera: Ceratopogonidae). Environmental Entomology 35, 1154-1160.

Schmidtmann, E.T., Bobian, R.J., Belden, R.P., 2000. Soil chemistries define aquatic habitats with immature populations of the *Culicoides variipennis* Complex (Diptera: Ceratopogonidae). Journal of Medical Entomology 37, 58-64.

Schmidtmann, E.T., Herrero, M.V., Green, A.L., Dargatz, D.A., Rodriquez, J.M., Walton, T.E., 2011. Distribution of *Culicoides sonorensis* (Diptera: Ceratopogonidae) in Nebraska, South Dakota, and North Dakota: clarifying the epidemiology of bluetongue disease in the northern Great Plains region of the United States. Journal of Medical Entomology 48, 634-643.

Schmidtmann, E.T., Jones, C.J., Gollands, B., 1980. Comparative host-seeking activity of *Culicoides* (Diptera: Ceratopogonidae) attracted to pastured livestock in central New York state, USA. Journal of Medical Entomology 17, 221-231.

Schneider, J.R., Chadee, D.D., Mori, A., Romero-Severson, J., Severson, D.W., 2011. Heritability and adaptive phenotypic plasticity of adult body size in the mosquito *Aedes aegypti* with implications for dengue vector competence. Infection, Genetics and Evolution 11, 11-16.

Schofield, C.J., Galvão, C., 2009. Classification, evolution, and species groups within the Triatominae. Acta Tropica 110, 88-100.

Schwenkenbecher, J., Mordue, A., Piertney, S., 2009. Phylogenetic analysis indicates that *Culicoides dewulfi* should not be considered part of the *Culicoides obsoletus* complex. Bulletin of Entomological Research 99, 371.

Sellers, R.F., Maarouf, A.R., 1989. Trajectory analysis and bluetongue virus serotype 2 in Florida 1982. Canadian Journal of Veterinary Research 53, 100-102.

Shelley, A., Coscarón, S., 2001. Simuliid blackflies (Diptera: Simuliidae) and ceratopogonid midges (Diptera: Ceratopogonidae) as vectors of *Mansonella ozzardi* (Nematoda: Onchocercidae) in northern Argentina. Memórias do Instituto Oswaldo Cruz 96, 451-458.

Shults, P., Borkent, A., Gold, R., 2016. The pupa of *Culicoides sonorensis* Wirth and Jones (Diptera: Ceratopogonidae)—First detailed description of this stage of the bluetongue virus vector. Annals of the Entomological Society of America, 1-39.

Slatkin, M., Hudson, R.R., 1991. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129, 555-562.

Smith, K.E., Stallknecht, D.E., 1996. *Culicoides* (Diptera: Ceratopogonidae) collected during epizootics of hemorrhagic disease among captive white-tailed deer. Journal of Medical Entomology 33, 507-510.

Smith, K.E., Stallknecht, D.E., Nettles, V.F., 1996a. Experimental infection of *Culicoides lahillei* (Diptera: Ceratopogonidae) with epizootic hemorrhagic disease virus serotype 2 (Orbivirus: Reoviridae). Journal of Medical Entomology 33, 117-122.

Smith, K.E., Stallknecht, D.E., Sewell, C.T., Rollor, E.A., Mullen, G.R., Anderson, R.R., 1996b. Monitoring of *Culicoides* spp. at a site enzootic for hemorrhagic disease in white-tailed deer in Georgia, USA. Journal of Wildlife Diseases 32, 627-642.

Spinelli, G.R., Ronderos, M.M., 1997. Description of the male of *Culicoides lahillei* and revalidation of *Culicoides debilipalpis* (Diptera: Ceratopogonidae). Transactions of the American Entomological Society 123, 303-309.

Stallknecht, D., Howerth, E., 2004. Epidemiology of bluetongue and epizootic haemorrhagic disease in wildlife: surveillance methods. Veterinaria Italiana 40, 203-207.

Stephens, M., Scheet, P., 2005. Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. The American Journal of Human Genetics 76, 449-462.

Stevens, G., McCluskey, B., King, A., O'Hearn, E., Mayr, G., 2015. Review of the 2012 epizootic hemorrhagic disease outbreak in domestic ruminants in the United States. PLoS ONE 10, e0133359.

Steverding, D., 2014. The history of Chagas disease. Parasites & Vectors 7, 1-8.

Sutherst, R.W., 2004. Global change and human vulnerability to vector-borne diseases. Clinical Microbiology Reviews 17, 136-173.

Swanson, D., 2012. Ecology and phylogeny of the biting-midge genus *Culicoides* (Diptera: Ceratopogonidae). Entomology, Soils, and Plant Sciences. Clemson University, Clemson, SC, USA.

Tabachnick, W.J., 1990. Genetic variation in laboratory and field populations of the vector of bluetongue virus, *Culicoides variipennis* (Diptera: Ceratopogonidae). Journal of Medical Entomology 27, 24-30.

Tabachnick, W.J., 1992. Genetic differentiation among populations of *Culicoides variipennis* (Diptera: Ceratopogonidae), the North American Vector of bluetongue virus. Annals of the Entomological Society of America 85, 140-147.

Tabachnick, W.J., 1996. *Culicoides variipennis* and bluetongue-virus epidemiology in the United States. Annual Review of Entomology 41, 23-43.

Tabachnick, W.J., 2004. *Culicoides* and the global epidemiology of bluetongue virus. Veterinaria Italiana 40, 145-150.

Tabachnick, W.J., 2010. Challenges in predicting climate and environmental effects on vector-borne disease episystems in a changing world. Journal of Experimental Biology 213, 946-954.

Tabachnick, W.J., Black, W.C., 1995. Making a case for molecular population genetic studies of arthropod vectors. Parasitology Today 11, 27-30.

Tabachnick, W.J., Holbrook, F.R., 1992. The *Culicoides variipennis* complex and the distribution of bluetongue viruses in the United States. Proceedings of the United States Animal Health Association 96, 207-212.

Tajima, F., 1989a. The effect of change in population size on DNA polymorphism. Genetics 123, 597-601.

Tajima, F., 1989b. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585-595.

Tanya, V.N., Greiner, E.C., Gibbs, E.P.J., 1992. Evaluation of *Culicoides insignis* (Diptera: Ceratopogonidae) as a vector of bluetongue virus. Veterinary Microbiology 32, 1-14.

Tarlinton, R., Daly, J., Dunham, S., Kydd, J., 2012. The challenge of Schmallenberg virus emergence in Europe. The Veterinary Journal 194, 10-18.

Tompkins, D.M., Carver, S., Jones, M.E., Krkošek, M., Skerratt, L.F., 2015. Emerging infectious diseases of wildlife: a critical perspective. Trends in Parasitology 31, 149-159.

Tosh, R.B., Chaniotis, B.N., Johnson, K.M., 1971. Vesicular stomatitus Indiana serotype: mulitiplication in and transmission by experimentally infected phlebotomine sandflies (*Lutzomyia trapidoi*) American Journal of Epidemiology 93, 491-495.

Valkiunas, G., 2004. Avian malaria parasites and other haemosporidia. CRC press, Boca Raton, Florida.

Vaughan, J.A., Turner, E.C., 1987. Development of immature *Culicoides variipennis* (Diptera: Ceratopogonidae) from Saltville, Virginia, at constant laboratory temperatures. Journal of Medical Entomology 24, 390-395.

Veronesi, E., Henstock, M., Gubbins, S., Batten, C., Manley, R., Barber, J., Hoffmann, B., Beer, M., Attoui, H., Mertens, P.P.C., Carpenter, S., 2013. Implicating *Culicoides* biting bidges as vectors of Schmallenberg virus using semi-quantitative rt-PCR. PLoS ONE 8, e57747.

Vigil, S.L., Wlodkowski, J.C., Parris, J., Edwards de Vargas, S., Shaw, D., Cleveland, C., Grogan Jr, W.L., Corn, J.L., 2014. New records of biting midges of the genus *Culicoides* Latreille from the southeastern United States (Diptera: Ceratopogonidae).

Waltari, E., Hoberg, E.P., Lessa, E.P., Cook, J.A., 2007. Eastward Ho: phylogeographical perspectives on colonization of hosts and parasites across the Beringian nexus. Journal of Biogeography 34, 561-574.

Walton, T.E., Webb, P.A., Kramer, W.L., Smith, G.C., Davis, T., Holbrook, F.R., Moore, C.G., Schiefer, T.J., Jones, R.H., Janney, G.C., 1987. Epizootic vesicular stomatitis in Colorado, 1982: epidemiologic and entomologic Studies. The American Journal of Tropical Medicine and Hygiene 36, 166-176.

Weaver, S.C., Barrett, A.D., 2004. Transmission cycles, host range, evolution and emergence of arboviral disease. Nature reviews Microbiology 2, 789-801.

Wenk, C.E., Kaufmann, C., Schaffner, F., Mathis, A., 2012. Molecular characterization of Swiss Ceratopogonidae (Diptera) and evaluation of real-time PCR assays for the identification of *Culicoides* biting midges. Veterinary Parasitology 184, 258-266.

Wheeler, Q.D., 2004. Taxonomic triage and the poverty of phylogeny. Philosophical Transactions of the Royal Society of London B: Biological Sciences 359, 571-583.

Wheeler, Q.D., Raven, P.H., Wilson, E.O., 2004. Taxonomy: impediment or expedient? Science 303, 285.

White, D.M., Wilson, W.C., Blair, C.D., Beaty, B.J., 2005. Studies on overwintering of bluetongue viruses in insects. Journal of General Virology 86, 453-462.

Wiesers-Chimpf, L., Wilson, W., French, D., Bahama, A., Foil, L., 1993. Bluetongue virus in sheep and cattle and *Culicoides variipennis* and *C. stellifer* (Diptera: Ceratopogonidae) in Louisiana. Journal of Medical Entomology 30, 719-724.

Wiethoelter, A.K., Beltrán-Alcrudo, D., Kock, R., Mor, S.M., 2015. Global trends in infectious diseases at the wildlife–livestock interface. Proceedings of the National Academy of Sciences 112, 9662-9667.

Wilson, A., Darpel, K., Mellor, P.S., 2008. Where does bluetongue virus sleep in the winter? PLoS Biology 6, e210.

Wilson, A.J., Mellor, P.S., 2009. Bluetongue in Europe: past, present and future. Philosophical Transactions of the Royal Society B: Biological Sciences 364, 2669-2681.

Wilson, W.C., Mecham, J.O., Schmidtmann, E.T., Sanchez, C.J., Herrero, M., Lager, I., 2009. Current status of bluetongue in the Americas. In: Mellor, P.S., Baylis, M., Mertens, P.P.C. (Eds.), Bluetongue. Academic Press, London, UK, pp. 197-222.

Wirth, W., Morris, C., 1985. The taxonomic complex, *Culicoides variipennis*. In: Barber, T., Jochim, M. (Eds.), Bluetongue and related orbiviruses, Liss, New York, pp. 165-175.

Wirth, W.W., Dyce, A.L., Peterson, B.V., Roper, I., 1985. An atlas of wing photographs, with a summary of the numerical characters of Nearctic species of *Culicoides* (Diptera : Ceratopogonidae). Contributions of the American Entomological Institute 22, 1-46.

Wirth, W.W., Jones, R.H., 1957. The North American subspecies of *Culicoides variipennis* (Diptera, Heleidae). United States Deptartment of Agriculture, Washington, D.C., U.S.A.

Wirth, W.W., Mullens, B.A., 1992. *Culicoides boydi* (Diptera: Ceratopogonidae): A potential vector of hemorrhagic disease viruses to desert bighorn sheep in Southern California. Journal of Medical Entomology 29, 1006-1010.

Work, T.M., Jessup, D.A., Sawyer, M.M., 1992. Experimental bluetongue and epizootic hemorrhagic disease virus infection in California black-tailed deer. Journal of wildlife diseases 28, 623-628.

Zwickl, D.J., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. University of Texas at Austin.

CHAPTER 3

Picky eaters are rare: DNA-based bloodmeal analysis of the *Culicoides variipennis* species complex (Diptera: Ceratopogonidae)

Introduction

Understanding the sylvatic cycles and predicting epizootics of vector-borne pathogens requires knowledge about disease vector feeding behavior. Elucidating vector and host interactions helps with discovery of unknown vector species, susceptible hosts, reservoir species, and host-parasite-vector co-evolution (e.g., Hamer et al., 2008; Malmqvist et al., 2004). One of the most critical ecological parameters for predicting vector-borne transmission is vector biting rates on susceptible hosts (Garrett-Jones, 1964; Kent, 2009; Lyimo and Ferguson, 2009). Accurate host identification is thus a requirement for determining the impact these pathogens may have on human, wildlife, and livestock health.

Sylvatic transmission pathways of vector-borne pathogens are difficult to delineate as they involve many aspects, including multiple host species. Many blood-feeding arthropods that serve as vectors, especially in the Diptera, have feeding behaviors that can be plastic in response to environmental change and host availability (Kilpatrick et al., 2007; Lefèvre et al., 2009). This complexity is further confounded by our lack of knowledge about diversity of host choice (Harrup et al., 2015; Parham et al., 2015; Purse et al., 2015). Disentangling vector-borne pathogen transmission networks for predicting future outbreaks requires full characterization of the breadth of host species upon which vectors feed (LoGiudice et al., 2003).

Biting midges of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) are globally ubiquitous, except for New Zealand and Antarctica, and the group includes over 1400 described species (Borkent, 2015; Mellor, 2000). Females of most species are hematophagous and transmit a multitude of pathogenic viruses, filarial worms, and blood parasites to humans, livestock, and wildlife (Borkent, 2005). Among the most damaging *Culicoides*-borne pathogens are bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), vesicular stomatitis virus, African horse sickness, and Schmallenberg virus (Linden et al., 2012; Mellor, 2000; Tarlinton et al., 2012). Each of these viruses cause outbreaks in wildlife and livestock globally and have been linked to severe economic losses due to both lost production and disease-related trade restrictions (Hoar et al., 2003; Hoogendam, 2007; Tabachnick, 1996; Wilson and Mellor, 2009). In North America, the most commonly encountered vector species of BTV is *C. sonorensis* Wirth and Jones, but the evidence for which species is the main vector of EHDV is ambiguous (Foster et al., 1977; Foster et al., 1963; Ruder et al., 2015; Chapter 1 and 2)

Multiple methods can be employed to identify the source of arthropod bloodmeals, but to date genetic techniques have proven to be the most accurate when applied to *Culicoides* (e.g., Garros et al., 2011; Pettersson et al., 2012). In North America, three studies have used molecular methods to identify the source of *Culicoides* bloodmeals (Mullens and Dada, 1992; Swanson and Turnbull, 2014; Tempelis and Nelson, 1971). However, these studies were conducted over limited geographic areas and the sample sizes were very small. Here, I advance the knowledge base of *Culicoides* host choice by sequencing two mitochondrial DNA loci from blood-engorged *Culicoides* collected from multiple regions across the U.S., including one area where BTV is enzootic in both livestock and wildlife (Jessup et al., 1990; Roug et al., 2012). The knowledge gained from this study will help illuminate the diversity of *Culicoides* hosts, which will help us

better understand pathogen transmission across the livestock/wildlife interface, and the natural history of this ubiquitous, abundant, and economically important group of insects.

Materials and Methods

Field collection and morphological species identification

Blood-engorged *Culicoides* individuals were captured opportunistically as part of surveillance efforts, or for other studies, from 2011 to 2014 across the U.S. (Chapter 2). Insects were captured with CDC mini light traps (Bioquip, USA) or New Jersey light traps baited with ultraviolet light and CO_2. Insects were stored on dry ice immediately after capture then stored at -80 ºC, or placed immediately in 99% ethanol. *Culicoides* were sorted from trap by-catch under stereomicroscopes. Frozen insects were sorted on a cold plate (Bioquip, USA). Species identification was first based on wing patterns following Wirth et al. (1985). For some subgenera and species groups, wing patterns are not species-diagnostic so I used combinations of morphological characters identified as diagnostic by Blanton and Wirth (1979), Battle and Turner (1971), and Holbrook et al. (2000) to identify *Culicoides* species to the lowest possible taxon (Chapter 2). I also included three blood-engorged individuals from the *C. sonorensis* USDA-ARS captivity colony to validate my bloodmeal identification assays. These individuals were fed domestic sheep (*Ovis aries* L.) blood.

Bloodmeal identification

Genomic DNA from individual blood-engorged *Culicoides* females was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany). Insects were extracted in a room dedicated to low-quantity DNA samples to reduce the chances of contamination. Each specimen was placed in a sterile petri dish and I separated the abdomen from the thorax with tools that

were sterilized by dipping in absolute ethanol and flamed with a Bunsen burner. The entire insect was placed in 300 µL of Qiagen buffer ATL. I then added 20 µL of proteinase K and incubated the samples overnight at 56°C. The following day the supernatant was transferred to a new 1.7 mL tube, leaving the exoskeleton of the insect in the initial tube. I added absolute ethanol to the exoskeleton and stored it at -20°C for slide-mounting for species identification. I used an automated extraction protocol "Isolation of DNA from forensic casework samples part B (purification)" designed for the QIAmp DNA investigator kit (Qiagen, Germany) on the QIAcube (Qiagen, Germany) to extract DNA from the supernatant. The final elution volume was adjusted to 50 µL. An extraction blank containing only reagents was included with each extraction set to monitor for contamination. DNA extracts were stored at -20°C until further processing.

To identify the origins of *Culicoides* bloodmeals, I followed Pettersson et al. (2012) and used general primers COI_short_F/COI_short_R and L14841/H15149 to amplify around 350 bp of vertebrate cytochrome oxidase I (COI) and cytochrome-b (cyt-b), respectively (Kocher et al., 1989; Townzen et al., 2008). For COI, the total reaction volume was 25 µL and contained 2.5 µL 10x buffer II, 1 mM of $MgCl_2$, 0.1 mM of each dNTP, 0.4 mM of each primer and 2 units of Amplitaq Gold and 2 µL of DNA extract. The thermocycler profile included an initial denaturing step at 95ºC for 15 m then 45 cycles of 94ºC for 30s, annealing at 50ºC for 45s, extension at 72ºC for 30 s and a final extension at 72ºC for 10 m. The cyt-b gene was amplified in a 25 µL reaction that contained 2.5 µL 10x buffer II, 1.5 mM of $MgCl_2$, 0.15 mM of each dNTP, 0.4 mM of each primer and 2 units of Amplitaq Gold and 2 µL of DNA extract. The cyt-b thermocyclyer profile was identical to the one for COI but had an annealing temperature of 52ºC. Negative controls were included with each PCR to monitor contamination. PCR success (amplification)

was evaluated by electrophoresing products on 2% agarose gels stained with ethidium bromide. Successful reactions were purified using ExoSAP-it (Affymetrix, U.S.A.). Cycle sequencing was performed using BigDye v3.1 using the manufacturer's recommended thermocycler program (Life Technologies, U.S.A) but with an annealing temperature of 52℃. The cycle sequence products were purified with Sephadex clean-up using either PrepEase Sequencing Dye Clean-up Kit (Affymetrix) or 96-well filter plates (Whatman, U.S.A). Sanger sequencing was performed on an Applied Biosystems Genetic Analyzer 3500*xl* (Life Technologies, U.S.A).

The sequences were edited and compared to GenBank via the BLAST function in GENEIOUS R7 (Biomatters, New Zealand), or to the Barcode of Life Database (BOLD; Ratnasingham and Hebert, 2007) to determine species identification. I used a threshold of 98% to determine the species identification of vertebrate DNA sequences. If the sequences did not match to a species within this threshold on GenBank or BOLD then I identified the specimen as the genus with the highest match. In some cases known geographical distributions of hosts were used to assign the host to species (e.g., identified as *Odocoileus* from California it must be black-tailed deer (*Odocoileus hemionus* Rafinesque)).

Results

Field collection and morphological species identification

I collected a total of 199 blood-engorged *Culicoides* from three states: 14 from New York; seven from South Carolina; and 178 from California (Table 1; Appendix 3), which represented five morphologically identified species and one species complex (Table 1). The *C. variipennis* (Coquillett) species complex accounted for 176 (88%) of the individuals.

Bloodmeal identification

The validation of the approach was successful in that I identified the blood source of the three captive *C. sonorensis* as *O. aries*, the species from which the blood was collected. I successfully identified the sources of bloodmeals from all 199 wild-caught individuals (Table 1; Appendix 1). Of the 199 successfully identified bloodmeals, 183 (92%) came from the *C. variipennis* species complex. Most of the *C. variipennis* species complex bloodmeals were from large ungulates and these included both wild species, black-tailed deer (n = 72; 39%), 5 domestic mammals (n = 80; 44%), and swine (*Sus scrofa* L.; n = 3; 2%) which could be either feral or domestic (Table 1; Figure 1). Eleven of the 185 (6%) were from black-tailed jackrabbit (*Lepus californicus* Gray) and 11 (6%) were from domestic dog (*Canis familiaris* L.). The remaining six individuals (3%) of the *C. variipennnis* species complex had fed upon emu (*Dromaius novaehollandiae* Latham).

Sixteen of the total 199 (8%) identified bloodmeals came from species not in the *C. variipennis* species complex: *C. bigutattus* (Coquillett), *C. crepuscularis* Malloch, *C. reevesi* Wirth, *C. stellifer* (Coquillett), and *C. utahensis* Fox. All of the non-*variipennis* bloodmeals were from deer (*Odocoileus* spp.), except one *C. crepuscularis* that fed on a house finch (*Haemorhous mexicanus* (Müller)). I also detected human DNA in some of the samples but these samples were excluded from further analysis as determining whether it was a bloodmeal taken from a human or contamination was not possible.

Discussion

The main finding from this study was that North American *Culicoides* host choice is broader than previously understood. Through the use of genetic approaches, I identified a

diversity of known hosts, including some that are highly susceptible to infection with BTV and EHDV, and expanded the knowledge base through detection of previously undocumented hosts. My results represent the most comprehensive dataset to date regarding Nearctic *Culicoides* host choice. The value of these data cannot be understated as one of the most important shortcomings in our knowledge of *Culicoides* biology is the lack of data regarding trophic behavior and host selection across diverse habitats (Martínez-de la Puente et al., 2015; Ruder et al., 2015).

Bloodmeal analysis

Comprehending the diversity of hosts in a particular area, including the previously documented hosts, can help us understand the influence of host availability and density on *Culicoides* host choice and pathogen transmission (Lyimo and Ferguson, 2009). I identified the source of bloodmeals from 199 *Culicoides* which resulted in the identification of 12 different host species (Table 1; Figure 1). Four of these mammalian species are susceptible to *Culicoides*-borne pathogens and have been previously identified as hosts for the *C. variipennis* species complex: horse (*Equus caballus* L.); cattle (*Bos taurus* L.); domestic sheep (*Ov. aries*); and white-tailed deer (*O. virginianus* (Zimmerman)) (Foster et al., 1977; Jones, 1959; Jones et al., 1972; Luedke et al., 1967). The other six mammalian species detected: domestic dog; domestic goat (*Capra hircus* L.); donkey (*E. asinus* L.); black-tailed jackrabbit; swine; and black-tailed deer have not been documented as hosts of the *C. variipennis* complex until this study. The detection of bloodmeals from hosts that are susceptible to infection with BTV and EHDV is further evidence of the role that the *C. variipennis* species complex plays in pathogen transmission across the U.S. and the potential presence of reservoir species.

The results presented here, combined with previous findings, confirmed that the *C. variipennis* species complex will feed upon mammals other than large ungulates, indicating that

they are opportunistic when choosing host species for bloodmeals. Tempelis and Nelson (1971) used bait animals and immunological approaches to identify bloodmeals of the *C. variipennis* species complex in California, but could only distinguish to host family (Bovidae and Leporidae). My data substantiated their findings as I found 31 individuals had fed upon cattle (Bovidae), and I documented, for the first time, this insect group feeding on black-tailed jackrabbits (Leporidae; Table 1). I was also able to document the first occurrence of the *C. variipennis* species complex feeding on domestic dog. Alexander et al. (1994) reported that African carnivores can be infected with BTV. Our confirmation of *Culicoides* feeding upon a canid species and a wild leporid suggest that small mammals and carnivores should be evaluated as potential contributors to the transmission cycles of BTV and EHDV.

The data I collected for this study provide the first evidence that the *C. variipennis* complex will take bloodmeals from black-tailed deer. Epidemiological data suggest that *C. variipennis* will bite black-tailed deer as Roug et al. (2012) detected considerable numbers of black-tailed deer in northern California that were seropositive for both BTV and EHDV. Bluetongue virus has also been documented as moving freely between deer and livestock in the same area when large numbers of the *C. variipennis* species complex are present (Jessup et al., 1990). One remarkable point is that black-tailed deer are often asymptomatic when infected with BTV and EHDV thus detecting outbreaks in this host species can be challenging (Work et al., 1992). Asymptomatic hosts can facilitate movement of pathogens across the landscape, and may even facilitate overwintering of the viruses. The combination of bloodmeal and epidemiological data provides additional evidence that black-tailed deer can contribute to maintenance of the BTV enzootic cycle in northern California.

The first evidence of the *C. variipennis* species complex feeding on a bird was discovered in this study (Table1). Emus are not native to North America but there are commercial farms and hobbyists, including northern California, that raise the species for meat or other purposes (California Department of Agriculture, personal communication). My detection of *Culicoides* bloodmeals from emus was unexpected and these results counter the assumption that the species in the *C. variipennis* complex feed exclusively on mammals. In fact, considering the size of emus, one could hypothesize that host choice in the *C. variipennis* species complex is not necessarily driven by phylogeny but rather by factors associated with body size, such as the larger emissions of CO_2 or volatile compounds. This hypothesis has been tested with mosquitoes and other *Culicoides* species using captive animals of different sizes and taxonomic groups (Koch and Axtell, 1979; Lyimo and Ferguson, 2009; Port et al., 1980). These studies rejected the strict mammal versus bird hypothesis in favor of body size driven host choice. Emus can be infected with avian blood parasites (Fox et al., 1996) and, if these animals are imported or transferred to locales with naïve hosts, they should be screened for infection to limit the chances of introduction and potential transmission of foreign blood parasite species to North American poultry and wild birds through *Culicoides* (Valkiunas, 2004).

I obtained bloodmeal identification for six other *Culicoides* species not in the *C. variipennis* complex (Table 1). *Culicoides biguttatus*, a common species east of the Mississippi River (Blanton and Wirth, 1979), fed on white-tailed deer, which confirms previous findings (Schmidtmann et al., 1980; Smith et al., 1996; Swanson, 2012; Zimmerman and Turner, 1983). In fact, one of these studies documented *C. biguttatus* feeding on white-tailed deer in an area that is enzootic for both BTV and EHDV which points to a potential role of this species as a vector (Smith et al., 1996). *Culicoides stellifer* is a common species across continental North America

(Blanton and Wirth, 1979) that I found had fed on white-tailed deer. This species has been documented as feeding on large ungulates and is suspected as playing a role in the transmission of EHDV (Ruder et al., 2015; Schmidtmann et al., 1980; Smith and Stallknecht, 1996; Smith et al., 1996). I collected one *C. crepuscularis,* a species that ranges across the continental U.S., and this individual had fed upon a house finch. This *Culicoides* species is known to prefer avian species and is also a vector for avian blood parasites and nematodes (Bennett, 1961; Fallis and Bennett, 1961a; Hoffman, 1925). *Culicoides utahensis* is found only in the western U.S. and is known to use deer as a bloodmeal source (Wirth and Rowley, 1971), which I confirmed. *Culicoides reevesi* is distributed in the southwestern U.S. and, to date, has only been recorded feeding on humans (Grogan Jr. et al., 2004). My data reflect the first record of *C. reevesi* feeding on black-tailed deer.

Conclusion

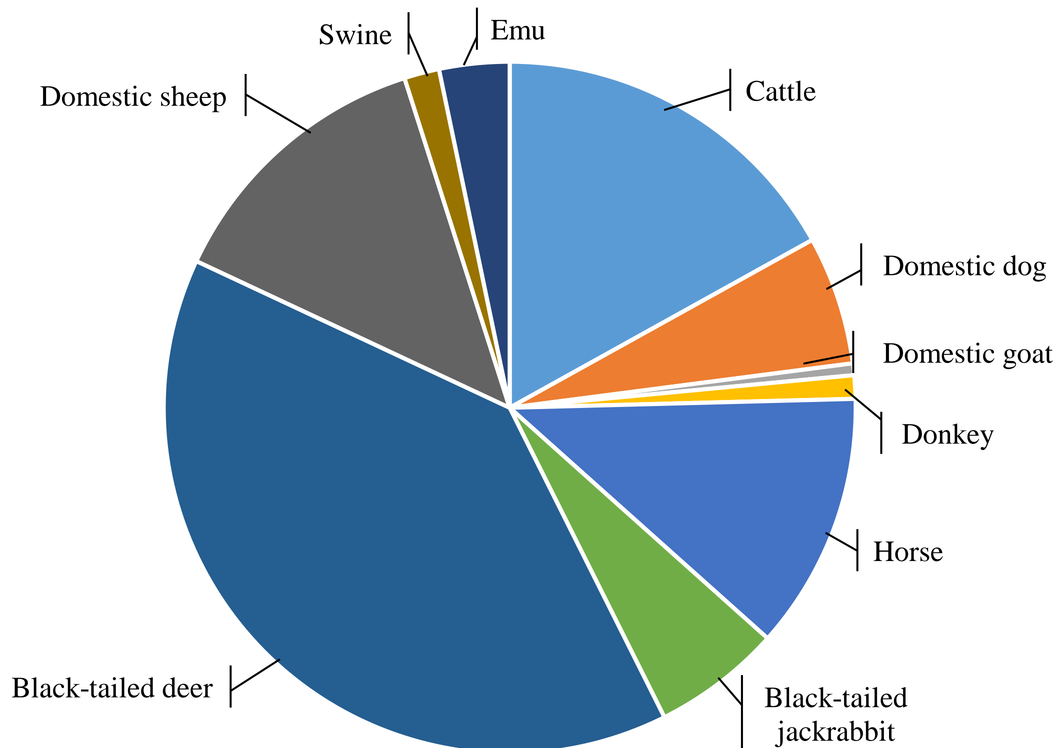
Vector-borne pathogens are significant threats to the health and welfare of both humans and animals worldwide (Mellor et al., 2000). More investigations into the ecology of all *Culicoides* species, not just the currently known vectors, will lead to better predictions of disease outbreaks and their attendant negative impacts on animal and human populations. In the face of climate change, the distributions of vector species and pathogens are expected to change (Harvell et al., 2002; Kovats et al., 2001). The potential exists for *Culicoides*-borne pathogens restricted to warmer parts of the world to move north or south leading to devastating outbreaks (Carpenter et al., 2009; Purse et al., 2005; Tabachnick, 2010). With the continuous threat of the introduction of foreign pathogens, we must build a solid knowledge base regarding *Culicoides* host choice so we can develop better models to predict transmission and develop methods to study and manage *Culicoides* and the pathogens they transmit. My dataset is the most comprehensive bloodmeal

analysis for North American *Culicoides* to date. I have demonstrated that molecular tools are important tools for gaining a solid knowledge base and developing methods to address some of the pressing needs for managing *Culicoides*-borne diseases.

Table 1. Results from molecular bloodmeal analyses based on the cytochrome oxidase I (COI) and the cytochrome-b (cyt-b) genes for *Culicoides* species in North America. Presented in the table are *Culicoides* species, the state where collected, sample size (n), number of host species identifications from bloodmeals. The host species are: Black-tailed deer (*Odocoileus hemionus*); Black-tailed jackrabbit (*Lepus californicus*); Cattle (*Bos taurus*); Domestic dog (*Canis familiaris*); Domestic goat (*Capra hircus*); Domestic sheep (*Ovis aries*); Donkey (*Equus asinus*); Horse (*Equus. caballus*); Swine (*Sus scofa*); White-tailed deer (*O. virginianus*); Emu (*Dromaius novaehollandiae)*; House finch (*Haemorhous mexicanus*).

			Mammals										Birds	
Culicoides species	State	n	Cattle	Domestic dog	Domestic goat	Donkey	Horse	Black-tailed jackrabbit	Black-tailed deer	White-tailed deer	Domestic sheep	Swine	Emu	House finch
C. biguttatus	New York	9								9				
C. crepuscularis	California	1												1
C. reevesi	California	1							1					
C. stellifer	New York	4								4				
C. utahensis	California	1							1					
C. variipennis complex	California	176	24	11	1	2	22	11	72		24	3	6	
C. variipennis complex	South Carolina	7	7											
Total		199	31	11	1	2	22	11	74	13	24	3	6	1

Figure 1. Pie chart representing the diversity and proportion of hosts identified from *Culicoides variipennis* species complex bloodmeals using two mitochondrial DNA loci. The host species are: Black-tailed deer (*Odocoileus hemionus*); Black-tailed jackrabbit (*Lepus californicus*); Cattle (*Bos taurus*); Domestic dog (*Canis familiaris*); Domestic goat (*Capra hircus*); Domestic sheep (*Ovis aries*); Donkey (*Equus asinus*); Horse (*Equus. caballus*); Swine (*Sus scofa*); White-tailed deer (*O. virginianus*); Emu (*Dromaius novaehollandiae)*; House finch (*Haemorhous mexicanus*).



LITERATURE CITED

Alexander, K., Maclachlan, N.J., Kat, P., House, C., O'Brien, S., Lerche, N., Sawyer, M., Frank, L., Holekamp, K., Smale, L., 1994. Evidence of natural bluetongue virus infection among African carnivores. American Journal of Tropical Medicine and Hygiene 51, 568-576.

Battle, F.V., Turner, E.C., 1971. A systematic review of the genus *Culicoides*:(Diptera: Ceratopogonidae) in Virginia, with a geographic catalog of the species occurring in the eastern United States north of Florida. Virginia Polytechnic Institute and State University.

Bennett, G.F., 1961. On the specificity and transmission of some avian trypanosomes. Canadian Journal of Zoology 39, 17-33.

Blanton, F.S., Wirth, W.W., 1979. The sand flies (*Culicoides*) of Florida (Diptera: Ceratopogonidae). Florida Department of Agriculture and Consumer Services, Gainesville, Florida, USA.

Borkent, A., 2005. The biting midges, the Ceratopogonidae (Diptera). In: Marquardt, W.C. (Ed.), Biology of Disease Vectors. Elsevier Academic Press, London, UK, pp. 113-126.

Borkent, A., 2015. World species of biting midges (Diptera: Ceratopogonidae). Royal British Columbia Museum, American Museum of Natural History, and Instituto Nacional de Biodiversidad, Salmon Arm, British Columbia, Canada.

Carpenter, S., Wilson, A., Mellor, P.S., 2009. *Culicoides* and the emergence of bluetongue virus in northern Europe. Trends in Microbiology 17, 172-178.

Fallis, A.M., Bennett, G.F., 1961. Ceratopogonidae as intermediate hosts for *Haemoproteus* and other parasites. Mosquito News 21, 21-28.

Foster, N., Breckon, R., Luedke, A., Jones, R., 1977. Transmission of two strains of epizootic hemorrhagic disease virus in deer by *Culicoides variipennis*. Journal of Wildlife Diseases 13, 9-16.

Foster, N., Jones, R., McCrory, B., 1963. Preliminary investigations on insect transmission of bluetongue virus in sheep. American Journal of Veterinary Research 24, 1195.

Fox, J.H., Greiner, E.C., Bain, P., Jones, R., 1996. Malaria in a captive Emu (*Dromaius novaehollandiae*) from Florida. Avian Diseases, 477-479.

Garrett-Jones, C., 1964. Prognosis for interruption of malaria transmission through assessment of the mosquito's vectorial capacity. Nature 204, 1173-1175.

Garros, C., Gardès, L., Allène, X., Rakotoarivony, I., Viennet, E., Rossi, S., Balenghien, T., 2011. Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae: Diptera): applications on Palaearctic biting midge species, vectors of Orbiviruses. Infection, Genetics and Evolution 11, 1103-1110.

Grogan Jr., W.L., Spinelli, G.R., Phillips, R.A., Woodward, D.L., 2004. The male of *Culicoides reevesi* Wirth, with a redescription of the female and new seasonal activity, distribution, and biting records (Diptera: Ceratopogonidae). Western North American Naturalist, 433-438.

Hamer, G.L., Kitron, U.D., Brawn, J.D., Loss, S.R., Ruiz, M.O., Goldberg, T.L., Walker, E.D., 2008. *Culex pipiens* (Diptera: Culicidae): A bridge vector of West Nile virus to humans. Journal of Medical Entomology 45, 125-128.

Harrup, L.E., Bellis, G.A., Balenghien, T., Garros, C., 2015. *Culicoides* Latreille (Diptera: Ceratopogonidae) taxonomy: Current challenges and future directions. Infection, Genetics and Evolution 30, 249-266.

Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., Samuel, M.D., 2002. Climate warming and disease risks for terrestrial and marine biota. Science 296, 2158-2162.

Hoar, B.R., Carpenter, T.E., Singer, R.S., Gardner, I.A., 2003. Regional risk of exporting cattle seropositive for bluetongue virus from the United States. American Journal of Veterinary Research 64, 520-529.

Hoffman, W., 1925. A review of the species of *Culicoides* of North and Central America and the West Indies. American Journal of Epidemiology 5, 274-301.

Holbrook, F.R., Tabachnick, W.J., Schmidtmann, E.T., McKinnon, C.N., Bobian, R.J., Grogan Jr, W.L., 2000. Sympatry in the *Culicoides variipennis* complex (Diptera: Ceratopogonidae): a taxonomic reassessment. Journal of Medical Entomology 37, 65-76.

Hoogendam, K., 2007. International study on the economic consequences of outbreaks of bluetongue serotype 8 in north-western Europe. Van Hall Institute, Leeuwarden, The Netherlands.

Jessup, D., Work, T., Bushnell, R., Sawyer, M., Osburn, B., 1990. An outbreak of bluetongue in captive deer and adjacent livestock in Kern County, California. California Fish and Game 76, 83-90.

Jones, R., 1959. *Culicoides* breeding in human sewage sites of dwellings in Texas. Mosquito News 19, 164-167.

Jones, R., Potter, H., Rhodes, H., 1972. Ceratopogonidae attacking horses in south Texas during the 1971 VEE epidemic. Mosquito News 32, 507-509.

Kent, R.J., 2009. Molecular methods for arthropod bloodmeal identification and applications to ecological and vector‐borne disease studies. Molecular Ecology Resources 9, 4-18.

Kilpatrick, A.M., Kramer, L.D., Jones, M.J., Marra, P.P., Daszak, P., Fonseca, D.M., 2007. Genetic influences on mosquito feeding behavior and the emergence of zoonotic pathogens. The American Journal of Tropical Medicine and Hygiene 77, 667-671.

Koch, H.G., Axtell, R.C., 1979. Attraction of *Culicoides furens* and *C. hollensis* (Diptera: Ceratopogonidae) to animal hosts in a salt marsh habitat. Journal of Medical Entomology 15, 494-499.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences 86, 6196-6200.

Kovats, R.S., Campbell-Lendrum, D.H., McMichel, A.J., Woodward, A., Cox, J.S.H., 2001. Early effects of climate change: do they include changes in vector-borne disease? Philosophical Transactions of the Royal Society of London B: Biological Sciences 356, 1057-1068.

Lefèvre, T., Gouagna, L.-C., Dabiré, K.R., Elguero, E., Fontenille, D., Renaud, F., Costantini, C., Thomas, F., 2009. Beyond nature and nurture: phenotypic plasticity in blood-feeding behavior of *Anopheles gambiae* s.s. when humans are not readily accessible. The American Journal of Tropical Medicine and Hygiene 81, 1023-1029.

Linden, A., Desmecht, D., Volpe, R., Wirtgen, M., Gregoire, F., Pirson, J., Paternostre, J., Kleijnen, D., Schirrmeier, H., Beer, M., Garigliany, M.-M., 2012. Epizootic spread of Schmallenberg virus among wild cervids, Belgium, Fall 2011. Emerging Infectious Diseases 18, 2006-2008.

LoGiudice, K., Ostfeld, R.S., Schmidt, K.A., Keesing, F., 2003. The ecology of infectious disease: Effects of host diversity and community composition on Lyme disease risk. Proceedings of the National Academy of Sciences 100, 567-571.

Luedke, A., Jones, R., Jochim, M., 1967. Transmission of bluetongue between sheep and cattle by *Culicoides variipennis*. American journal of veterinary research 28, 457-460.

Lyimo, I.N., Ferguson, H.M., 2009. Ecological and evolutionary determinants of host species choice in mosquito vectors. Trends in Parasitology 25, 189-196.

Malmqvist, B., Strasevicius, D., Hellgren, O., Adler, P.H., Bensch, S., 2004. Vertebrate host specificity of wild–caught blackflies revealed by mitochondrial DNA in blood. Proceedings of the Royal Society of London B: Biological Sciences 271, S152-S155.

Martínez-de la Puente, J., Figuerola, J., Soriguer, R., 2015. Fur or feather? Feeding preferences of species of *Culicoides* biting midges in Europe. Trends in Parasitology 31, 16-22.

Mellor, P.S., 2000. Replication of Arboviruses in Insect Vectors. Journal of Comparative Pathology 123, 231-247.

Mellor, P.S., Boorman, J., Baylis, M., 2000. *Culicoides* biting midges: their role as arbovirus vectors. Annual Review of Entomology 45, 307-340.

Mullens, B.A., Dada, C.E., 1992. Spatial and seasonal distribution of potential vectors of hemorrhagic disease viruses to peninsular bighorn sheep in the Santa Rosa mountains of southern California. Journal of Wildlife Diseases 28, 192-205.

Parham, P.E., Waldock, J., Christophides, G.K., Hemming, D., Agusto, F., Evans, K.J., Fefferman, N., Gaff, H., Gumel, A., LaDeau, S., Lenhart, S., Mickens, R.E., Naumova, E.N., Ostfeld, R.S., Ready, P.D., Thomas, M.B., Velasco-Hernandez, J., Michael, E., 2015. Climate, environmental and socio-economic change: weighing up the balance in vector-borne disease transmission. Philosophical Transactions of the Royal Society of London B: Biological Sciences 370.

Pettersson, E., Bensch, S., Ander, M., Chirico, J., Sigvald, R., Ignell, R., 2012. Molecular identification of bloodmeals and species composition in *Culicoides* biting midges. Medical and Veterinary Entomology 27, 104-112.

Port, G.R., Boreham, P.F.L., Bryan, J.H., 1980. The relationship of host size to feeding by mosquitoes of the *Anopheles gambiae* Giles complex (Diptera: Culicidae). Bulletin of Entomological Research 70, 133-144.

Purse, B.V., Carpenter, S., Venter, G.J., Bellis, G., Mullens, B.A., 2015. Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of *Culicoides*-borne viruses. Annual Review of Entomology 60, 373-392.

Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P.C., Baylis, M., 2005. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Micro 3, 171-181.

Ratnasingham, S., Hebert, P.D., 2007. BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). Molecular ecology notes 7, 355-364.

Roug, A., Swift, P., Torres, S., Jones, K., Johnson, C.K., 2012. Serosurveillance for livestock pathogens in free-ranging mule deer (*Odocoileus hemionus*). PLoS ONE 7, e50600.

Ruder, M.G., Lysyk, T.J., Stallknecht, D.E., Foil, L.D., Johnson, D.J., Chase, C.C., Dargatz, D.A., Gibbs, E.P.J., 2015. Transmission and epidemiology of bluetongue and epizootic

hemorrhagic disease in North America: Current perspectives, research gaps, and future directions. Vector-Borne and Zoonotic Diseases 15, 348-363.

Schmidtmann, E.T., Jones, C.J., Gollands, B., 1980. Comparative host-seeking activity of *Culicoides* (Diptera: Ceratopogonidae) attracted to pastured livestock in central New York state, USA. Journal of Medical Entomology 17, 221-231.

Smith, K.E., Stallknecht, D.E., 1996. *Culicoides* (Diptera: Ceratopogonidae) collected during epizootics of hemorrhagic disease among captive white-tailed deer. Journal of Medical Entomology 33, 507-510.

Smith, K.E., Stallknecht, D.E., Sewell, C.T., Rollor, E.A., Mullen, G.R., Anderson, R.R., 1996. Monitoring of *Culicoides* spp. at a site enzootic for hemorrhagic disease in white-tailed deer in Georgia, USA. Journal of Wildlife Diseases 32, 627-642.

Swanson, D.A., Turnbull, M.W., 2014. Molecular Identification of Bloodmeals from *Culicoides* Latreille (diptera: Ceratopogonidae) in the Southeastern U.S.A. Proceedings of the Entomological Society of Washington 116, 354-357.

Tabachnick, W.J., 1996. *Culicoides variipennis* and bluetongue-virus epidemiology in the United States. Annual Review of Entomology 41, 23-43.

Tabachnick, W.J., 2010. Challenges in predicting climate and environmental effects on vector-borne disease episystems in a changing world. Journal of Experimental Biology 213, 946-954.

Tarlinton, R., Daly, J., Dunham, S., Kydd, J., 2012. The challenge of Schmallenberg virus emergence in Europe. The Veterinary Journal 194, 10-18.

Tempelis, C.H., Nelson, R.L., 1971. Blood-feeding patterns of midges of the *Culicoides variipennis* comples in Kern County, California. Journal of Medical Entomology 8, 532-534.

Townzen, J.S., Brower, A.V.Z., Judd, D.D., 2008. Identification of mosquito bloodmeals using mitochondrial cytochrome oxidase subunit I and cytochrome b gene sequences. Medical and Veterinary Entomology 22, 386-393.

Valkiunas, G., 2004. Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton, Florida.

Wilson, A.J., Mellor, P.S., 2009. Bluetongue in Europe: past, present and future. Philosophical Transactions of the Royal Society B: Biological Sciences 364, 2669-2681.

Wirth, W.W., Dyce, A.L., Peterson, B.V., Roper, I., 1985. An atlas of wing photographs, with a summary of the numerical characters of Nearctic species of *Culicoides* (Diptera : Ceratopogonidae). Contributions of the American Entomological Institute 22, 1-46.

Wirth, W.W., Rowley, W.A., 1971. A revision of the *palmerae* group of the genus *Culicoides*. Journal of the Kansas Entomological Society, 153-171.

Work, T.M., Jessup, D.A., Sawyer, M.M., 1992. Experimental bluetongue and epizootic hemorrhagic disease virus infection in California black-tailed deer. Journal of wildlife diseases 28, 623-628.

Zimmerman, R.H., Turner, E.C., 1983. Host-feeding patterns of *Culicoides* (Diptera: Ceratopogonidae) collected from livestock in Virginia, USA. Journal of Medical Entomology 20, 514-519.

APPENDICES

Appendix 1-Metadata for *Culicoides* individuals collected across the United States. These samples were used for DNA sequencing and phylogenetic analyses. Included in the appendix are: state; name of collection site; coordinates; morphological species identification, sex of sample, number of samples per site, per each clade, haplogroup, and cluster (n); clade from gene trees that included the samples; haplotype group in median-joining networks that included the samples (haplogroup); and genetic cluster membership as identified in BAPS (cluster). The data per clade, haplogroup, and cluster are represented for each individual genetic locus: COI; COII; CAD; and TPI.

		Coordinates				COI			COII			CAD				TPI			
State	Site	Latitude	Longitude	Morphospecies	sex	n	clade	haplogroup	n	clade	haplogroup	n	clade	haplogroup	cluster	n	clade	haplogroup	cluster
AL	#2	31.6939	-87.7979	*C. sonorensis*	Female	12	I	COI-A	6	I	COII-A	8	I	CAD-A	CAD-1, CAD-2	7	I	TPI-A	TPI-3
AZ	Watson Lake	34.5792	-112.4258	*C. sonorensis*	Female	7	I	COI-A	3	I	COII-A	6	I	CAD-A	CAD-2	6	I	TPI-A	TPI-1
	WR25	34.7316	-112.5101	*C. sonorensis*	Female	5	I	COI-A	5	I	COII-A	4	I	CAD-A	CAD-2				
BC	W Kamloops	50.6630	-120.5040	*C. sonorensis*		1	I	COI-A											
	W Kamloops	50.6630	-120.5040	*C. sonorensis*		11	III	COI-D											
CA	LC-1	39.0166	-122.6585	*C. sonorensis* or *C. occidentalis*	Female	4	II, II	COI-C, COI-D					I	CAD-A		2	II	TPI-B	
	LC-2	38.9718	-122.6608	*C. sonorensis* or *C. occidentalis*	Female	3	II	COI-B	1	II	COII-B	1	II	CAD-B		3	II	TPI-B	
	Big Borax Lake	38.9841	-122.6646	*C. occidentalis*	Male	4	I, II	COI-A, COI-B	5	II	COII-B	3	II	CAD-B		5	II	TPI-B	
	San Diego 7	32.5508	-117.0836	*C. sonorensis* or *C. occidentalis*	Female	2	III	COI-D	4	III	COII-C	4	I, II	CAD-A, CAD-B	CAD-2				
	San Diego 59	33.0069	-117.2472	*C. sonorensis* or *C. occidentalis*	Female	5	I, II	COI-A, COI-C	3	I	COII-A	2	I	CAD-A	CAD-2	4	I	TPI-A	TPI-1
	San Diego 77	33.0222	-117.2444	*C. sonorensis* or *C. occidentalis*	Female	1	III	COI-D				2	I, II	CAD-A, CAD-B	CAD-2				
	Ventura	34.2010	-119.0010	*C. occidentalis*		1	II	COI-B											
CO	Fort Collins	40.5812	-105.1399	*C. sonorensis*	Female	3	I	COI-A	2	I	COII-A	2	I	CAD-A	CAD-2	4	I	TPI-A	TPI-1, TPI-3
	Fort Collins-Dixon Reservoir	40.5534	-105.1433	*C. sonorensis*	Female	1	I	COI-A								1	I	TPI-A	TPI-3
	Fort Collins-Bison Pens	40.5821	-105.1459	*C. sonorensis*	Female				1	I	COII-A	1	I	CAD-A	CAD-2	1	I	TPI-A	TPI-3
	Grand Junction	39.0546	-108.5170	*C. sonorensis*	Female	19	I, III	COI-A, COI-D	10	I	COII-A	10	I	CAD-A	CAD-2	6	I	TPI-A	TPI-1
	La Junta	37.7316	-103.6392	*C. sonorensis*	Female				3	I	COII-A	5	I, II	CAD-A, CAD-B	CAD-2	5	I	TPI-A	TPI-1, TPI-3
	Sylvan Dale COCDC113	40.4333	-105.2167	*C. sonorensis*	Female	1	I	COI-A				1	I	CAD-A	CAD-2				
ID	162AA	43.6356	-116.4388	*C. sonorensis*	Female	5	I, III	COI-A, COI-D	3	I, III	COII-A, COII-C	3	I	CAD-A	CAD-2	5	I, II	TPI-A, TPI-B	TPI-1
	183AA	43.7111	-116.4804	*C. sonorensis*	Female	4	I	COI-A	1	I	COII-A	5	I	CAD-A	CAD-2	5	I	TPI-A	TPI-1
	221AA	43.6492	-116.2973	*C. sonorensis*	Female	3	I	COI-A	3	I	COII-A	5	I	CAD-A	CAD-1, CAD-2	5	I	TPI-A	TPI-1
IL	Oak 1	40.1180	-89.9801	*C. variipennis*	Female	2	I	COI-A								2	I	TPI-A	TPI-2
IN	1			*C. variipennis*	Female	1	I	COI-A								1	I	TPI-A	TPI-2
	2			*C. variipennis*	Female	3	I	COI-A	2	I	COII-A					2	I	TPI-A	TPI-2
	6			*C. variipennis*	Female	2	I	COI-A				2	I	CAD-A	CAD-1	2	I	TPI-A	TPI-2
KS	Kloft North	39.2678	-97.1652	*C. sonorensis, C. variipennis*	Female	3	I	COI-A	1	I	COII-A					3	I	TPI-A	TPI-1, TPI-2
	Kloft South	39.2649	-97.1633	*C. variipennis*	Female	2	I	COI-A	1	I	COII-A					2	I	TPI-A	TPI-2, TPI-3
LA	Dairy Imp	30.4286	-91.1264	*C. sonorensis*	Female	8	I	COI-A	8	I	COII-A	4	I	CAD-A	CAD-2	5	I	TPI-A	TPI-1
	St. Gabriel	30.5545	-91.1586	*C. sonorensis, C. variipennis*	Female	8	I	COI-A	6	I	COII-A	5	I	CAD-A	CAD-1, CAD-2	8	I	TPI-A	TPI-1, TPI-2
MI	Midland	43.6544	-84.2575	*C. variipennis*	Female	6	I	COI-A	6	I	COII-A	4	I	CAD-A	CAD-2	5	I	TPI-A	TPI-3
MO	#5	38.8595	-91.8999	*C. variipennis*	Female	3	I	COI-A	4	I	COII-A	4	I	CAD-A	CAD-1	2	I	TPI-A	TPI-2
MT	Big Wall	46.5634	-108.3757	*C. sonorensis*	Female	2	I	COI-A	1	I	COII-A	1	I	CAD-A	CAD-2	2	I	TPI-A	TPI-3
	Gage Dome	46.5634	-108.3757	*C. sonorensis*	Female	4	I	COI-A	3	I	COII-A	4	I	CAD-A	CAD-2	5	I	TPI-A	TPI-3
NC	NCCDC2	35.9448	-79.6118	*C. variipennis*	Female	1	I	COI-A	1	I	COII-A	2	I	CAD-A	CAD-1	1	I	TPI-A	TPI-2
	NCCDC4	35.9487	-79.6137	*C. sonorensis*	Female	3	I	COI-A	2	I	COII-A	2	I	CAD-A	CAD-2	4	I	TPI-A	TPI-1
	NCCDC5	36.1731	-79.5596	*C. variipennis*	Female	5	I	COI-A	2	I	COII-A	5	I	CAD-A	CAD-1	2	I	TPI-A	TPI-1, TPI-2
ND	Belland 1	46.4169	-102.8225	*C. sonorensis*	Female	5	I	COI-A	5	I	COII-A	4	I	CAD-A	CAD-2	5	I	TPI-A	TPI-3
NE	Col.2	41.3345	-102.1480	*C. sonorensis*	Female											1	I	TPI-A	TPI-1
NJ	A	40.7781	-75.1112	*C. variipennis*	Female	5	I	COI-A	2	I	COII-A	5	I	CAD-A	CAD-1, CAD-2	4	I	TPI-A	TPI-3
	B	40.7808	-75.1219	*C. variipennis*	Female	4	I	COI-A	3	I	COII-A					3	I	TPI-A	TPI-2, TPI-3
NM	Canadian-R2	36.0709	-104.3482	*C. sonorensis or C. occidentalis*	Female	2	I, III	COI-A, COI-D	5	III	COII-C	7	I, II	CAD-A, CAD-B	CAD-2	5	I	TPI-A	TPI-1
	WRNWR2	35.8411	-105.0547	*C. sonorensis or C. occidentalis*	Female				5	I	COII-A	4	I	CAD-A	CAD-2	3	I	TPI-A	TPI-1
NV	Reno	39.4259	-119.7500	*C. sonorensis or C. occidentalis*	Female	2	I	COI-A	2	II	COI-B	6	I	CAD-A	CAD-2	5	I, II	TPI-A, TPI-B	TPI-1
	Sparks 10	39.6198	-119.7175	*C. sonorensis or C. occidentalis*	Female	3	I	COI-A	2	I	COII-A	1	I	CAD-A	CAD-2				
OH	Marks MI	42.5583	-84.7176	*C. variipennis*	Female	1	I	COI-A	1	I	COII-A								
	OSU Airport Dairy Barn	40.0844	-83.0876	*C. variipennis*	Female	10	I	COI-A	12	I	COII-A	19	I	CAD-A	CAD-1				
PA	#4	40.7525	-75.3066	*C. variipennis*	Female	2	I	COI-A	2	I	COII-A	2	I	CAD-A	CAD-1				
	Hummel Farm	40.3647	-77.0425	*C. variipennis*	Female	5	I	COI-A	8	I	COII-A	14	I	CAD-A	CAD-1				
SC	SCCDC2	34.4001	-81.6116	*C. variipennis*	Female				1	I	COII-A					1	I	TPI-A	TPI-2
	SCCDC4	34.3075	-81.7552	*C. variipennis, C. sonorensis*	Female	7	I	COI-A	11	I	COII-A	6	I	CAD-A	CAD-1, CAD-2	10	I	TPI-A	TPI-1, TPI-2
TX	#1	27.4424	-97.8138	*C. sonorensis*	Female	4	I	COI-A	3	I	COII-A	3	I	CAD-A	CAD-2	1	I	TPI-A	TPI-1
	#3	28.8324	-96.9240	*C. variipennis*	Female	14	I	COI-A	13	I	COII-A	19	I	CAD-A	CAD-1, CAD-2	12	I	TPI-A	TPI-2
VA	VACDC2	37.2302	-80.4818	*C. sonorensis*	Female	1	I	COI-A				1	I	CAD-A	CAD-2				
WY	PR1	44.1762	-106.1377	*C. sonorensis*	Female	3	I	COI-A	4	I	COII-A	2	I	CAD-A	CAD-2	2	I	TPI-A	TPI-3
	S2	44.0514	-107.9617	*C. sonorensis*	Female	1	I	COI-A	2	III	COII-C								
	TB1	44.0543	-104.6406	*C. sonorensis*	Female				2	I	COII-A								

Appendix 2-PCR conditions for each genetic locus that was amplified and sequenced from *Culicoides* samples for phylogenetic analyses. Provided in the appendix are the genetic locus, the primers used in the PCR, the citation from which the primer sequences were obtained and the thermocycler profile was adapted, the brand of taq polymerase, the reagents used in the PCR, the concentration of each of the reagents, and the thermocycler profile.

Locus	Primers	Citation	Taq polymerase	Reagent	Concentration	Thermocycler profile
COI	BC1culicFm	Bellis et al., (2013)	Amplitaq Gold (Life Technologies)	Total volume	25 µL	95° 15 m // 1x
	C1-N-2191	Dallas et al., (2003)		Buffer 10x	2.5 µL	94° 30s / 54° 30s / 72° 1 m// 40x
				MgCl2	2.5 mM	72° 5 m // 1x
				dNTP	0.25 mM	4° ∞
				Primer F	0.4 µM	
				Primer R	0.4 µM	
				Amplitaq Gold	2 Units	
				DNA	1 µL	
COII	TL-J3043	Beckenbach and Borkent (2003)	Amplitaq Gold (Life Technologies)	Total volume	25 µL	95° 15 m // 1 X
	TK-N3785	Beckenbach and Borkent (2003)		Buffer 10x	2.5 µL	94° 40s / 48° 1m / 72° 1m// 40x
				MgCl2	2 mM	72° 5 m // 1 X
				dNTP	0.2 mM	4° ∞
				Primer F	0.4 µM	
				Primer R	0.4 µM	
				Amplitaq Gold	2 Units	
				DNA	1 µL	
CAD	787F	Moulton and Wiegmann, (2004)	Amplitaq Gold (Life Technologies)	Total volume	15 µL	95° 15 m // 1 X
	CAD-4Rm	Bellis et al., (2013)		Buffer 10x	1.5 µL	94° 30s / 55° 30s / 72° 1 m// 5x
				MgCl2	2.8 mM	94° 30s / 50° 30s / 72° 1 m// 35x
				dNTP	0.4 mM	72° 5 m // 1 X
				Primer F	0.3 µM	4° ∞
				Primer R	0.3 µM	
				Amplitaq Gold	2 Units	
				DNA	2 µL	
CAD semi-nested	806Fm	Moulton and Wiegmann, (2004)	Amplitaq Gold (Life Technologies)	Total volume	15 µL	95° 15 m // 1 X
	CAD-4Rm	Bellis et al., (2013)		Buffer 10x	1.5 µL	94° 30s / 55° 30s / 72° 1 m// 5x
				MgCl2	2.8 mM	94° 30s / 50° 30s / 72° 1 m// 30x
				dNTP	0.4 mM	72° 5 m // 1 X
				Primer F	0.3 µM	4° ∞
				Primer R	0.3 µM	
				Amplitaq Gold	0.5 Units	
				PCR product	1 µL	
TPI	TPI-111fb	Bertone et al., (2008)	Illustra PuReTAQ ready-to-go PCR beads (GE Healthcare)	Total volume	25 µL	94° 5 m // 1 X
	277R	Bertone et al., (2008)		Primer F	0.3 µM	94° 30s / 52° 45s / 68° 2 m// 5x
				Primer R	0.3 µM	94° 30s / 51° 45s / 68° 2 m// 5x
				DNA	2 µL	94° 30s / 45° 45s / 68° 2.5 m// 36x
						68° 5 m // 1 X
						4° ∞

Appendix 3-Summary of data for blood-engorged *Culicoides* species collected in North America. Provided are the collection locality information (State, County, Latitude, and Longitude), year collected (year), *Culicoides* species, sample size at each locality (n), and identification of host using DNA-based bloodmeal analysis (Host, ID)

State	County	Year	Latitude	Longitude	*Culicoides* species	n	Host ID
CA	Lake	2010	38.945	-122.657	*C. variipennis* complex	3	black-tailed deer
CA	Lake	2010	38.971	-122.661	*C. variipennis* complex	2	black-tailed deer, cattle
CA	Lake	2010	38.990	-122.670	*C. variipennis* complex	35	black-tailed deer, black-tailed jackrabbit, cattle, dog, domestic goat, emu
CA	Lake	2010	38.990	-122.684	*C. variipennis* complex	4	black-tailed deer
CA	Lake	2013	39.017	-122.659	*C. variipennis* complex	21	black-tailed deer, black-tailed jackrabbit, cattle, domestic sheep
CA	Lake	2013	39.023	-122.920	*C. utahensis*	1	black-tailed deer
CA	Lake	2014	38.768	-122.520	*C. reevesi* , C. *variipennis* complex	6	black-tailed deer, cattle, domestic sheep, horse
CA	Lake	2014	38.777	-122.554	*C. variipennis* complex	5	black-tailed jackrabbit, cattle, horse
CA	Lake	2014	38.918	-122.589	*C. variipennis* complex	4	horse
CA	Lake	2014	38.919	-122.777	*C. variipennis* complex	1	horse
CA	Lake	2014	38.921	-122.625	*C. variipennis* complex	4	black-tailed deer, horse, swine
CA	Lake	2014	38.940	-122.658	*C. variipennis* complex	4	black-tailed deer, dog
CA	Lake	2014	38.941	-122.658	*C. variipennis* complex	4	cattle, dog, horse
CA	Lake	2014	38.990	-122.686	*C. variipennis* complex	5	black-tailed deer, cattle, swine
CA	Lake	2014	39.011	-122.656	*C. variipennis* complex	3	cattle, domestic sleep
CA	Lake	2014	39.013	-122.655	*C. variipennis* complex	7	cattle, domestic sleep
CA	Lake	2014	39.023	-122.921	*C. variipennis* complex	1	domestic sheep
CA	Lake	2014	39.058	-122.594	*C. variipennis* complex	3	black-tailed deer, black-tailed jackrabbit
CA	Lake	2014	39.066	-122.587	*C. variipennis* complex	17	black-tailed deer, black-tailed jackrabbit, dog, domestic sheep, donkey, horse, swine
CA	Lake	2014	39.074	-122.595	*C. variipennis* complex	7	black-tailed deer, dog, horse
CA	Lake	2014	39.075	-122.601	*C. variipennis* complex	11	black-tailed deer, dog, horse
CA	Lake	2014	39.146	-122.912	*C. variipennis* complex	1	black-tailed deer
CA	Lake	2013, 2014	38.972	-122.661	C. *variipennis* complex, *C. crepuscularis*	15	black-tailed deer, cattle, dog, house finch
CA	Lake	2013, 2014	38.984	-122.664	*C. variipennis* complex	15	black-tailed deer, black-tailed jackrabbit, cattle
NY	Chatham	2013			*C. biguttatus* , *C. stellifer*	13	white-tailed deer
SC	Newberry	2014	34.308	-81.755	*C. variipennis* complex	7	cattle