A STUDY OF *CULICOIDES* BITING MIDGES IN THE SUBGENUS *MONOCULICOIDES*: POPULATION GENETICS, TAXONOMY, SYSTEMATICS, AND CONTROL

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2021

Major Subject: Entomology

ABSTRACT

Only a small species of *Culicoides* midges are pathogen vectors in both managed and natural systems, and as such, proper species delimitation is vital. Before this study, the *C. variipennis* species complex contained three recognized species (only one of which is a vector; *C. sonorensis*), but limited molecular and morphological differences have hindered surveillance efforts. Single nucleotide polymorphism (SNP) data were generated using ddRAD sequencing for 206 individuals throughout the United States and Canada. Clustering analyses of these SNPs suggested the occurrence of at least two additional species in both sympatric and allopatric populations. The *C. variipennis* complex belongs to the subgenus *C.* (*Monoculicoides*), and here I present morphological, ecological, and molecular evidence in support of the taxonomic arrangement within this group. Two former synonyms were raised to full species status, four species were designated as new synonyms, and two new species were described. Keys to the adults of both sexes, bionomic information, and taxonomic discussions for all 26 species were provided. Despite some ecological overlap between many species of *C.* (*Monoculicoides*), only *C. sonorensis* appears to have any significant role in viral pathogen transmission. Identifying the molecular mechanisms behind the vector competency of this species will undoubtably lead to more options for control strategies. Next-generation control methods such as *Wolbachia*- and genetic-based population suppression and replacement are being investigated in other vector groups, and here we assess the feasibility and applicability of these approaches for use against biting midges.

DEDICATION

First and foremost, I dedicate this dissertation to my wife, Arli, whose love and patience have allowed me to have a career in a subject about which I am passionate. I would also not be where I am today without support and guidance from my parents, my brother, and my extended family. I was also lucky enough to marry into a wonderful family who have been encouraging.

My time at Texas A&M University has been wonderful, and much of that is thanks to the department of Entomology. Specifically, I would like to thank my advisor, Ed Vargo, for allowing me to have the opportunity to work full-time while also pursuing a doctoral degree. I truly appreciate that I was given the ability to study questions in which I was truly interested. I could not have asked for a better committee whose guidance has helped me achieve success, especially Art Borkent, who has been a mentor to me for both of my graduate degrees. His input and teachings have been invaluable, and I look forward to a long tenure as colleagues and friends.

Finally, a huge thanks to my coworkers, classmates, coworkers, and friends who have all supported and helped me along my doctoral journey. It is impossible to thank them all, but that in no way lessens my gratitude.

ACKNOWLEDGEMENTS

Chapter II

I would like to thank Art Borkent, Adam Jewess-Gaines, Dustin Swanson, Bonnie Ryan, Nadja Mayerle, multiple vector control agencies, USDA-APHIS-Wildlife Services, and several landowners for access to property or assisting with collecting specimens used in this study.

Chapter III

I would like to thank Torsten Dikow and Jim Pecor (USNM), Jeff Cumming (CNCI), Duncan Sivell (NHMUK) Bill Grogan, Bob Phillips, Bilal Dik, Heron Huerta for providing slide mounted material. Andrey Przhiboro and Jon Gelhaus for providing both slide mounted and fresh material. Gert Venter and Karien Labuschagne for providing fresh *C. cornutus* and Glenn Bellis for providing fresh *C. homotomus*.

Chapter IV

I would like to thank Alyssa Krafsur for helping to acquire images of *D. melanogaster*, *C. sonorensis*, and *Ae. albopictus* eggs. We would also like to thank the editor and two anonymous reviewers for their helpful comments and suggestions concerning the manuscript.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor Edward Vargo [advisor], Gabriel Hamer, and Hojun Song of the Department of Entomology, Professor Mariana Mateos of the Department of Ecology and conservation biology, and Art Borkent as a special appointment.

Funding Sources

Graduate study was supported by a fellowship from Texas A&M University and a dissertation research fellowship from the Urban Entomology Endowment fund. This work was also made possible in part by a USDA Non-Assistance Cooperative Agreement: 58-3020-9-007 and a S.W. Williston Diptera Research Award. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Texas A&M University.

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iii

ACKNOWLEDGEMENTS iv

CONTRIBUTORS AND FUNDING SOURCES v

TABLE OF CONTENTS vi

LIST OF FIGURES vii

LIST OF TABLES viii

I: INTRODUCTION: *CULICOIDES* BITING MIDGES & EVOLUTION 1

1.1 Species Delimitation 1
1.2 *Culicoides* Biting Midges 3
1.3 Overview 5

II: SPECIATION IN THE FACE OF LONG-RANGE DISPERSAL 8

2.1 Materials and Methods 11
2.2 Results 14
2.3 Discussion 18

III: A TAXONOMIC REVISION OF THE SUBGENUS *C.* (*MONOCULICOUDES*) 37

3.1 Materials and Methods 39
3.2 Taxonomic Discussion 42
3.3 Conclusion 151

IV: NEXT-GENERATION TOOLS TO CONTROL BITING MIDGE POPULATIONS 176

4.1 Management tools 178
4.2 Research gaps concerning novel control approaches against *Culicoides* 184
4.3 Engagement and risk assessment 192
4.4 Conclusions 194

V: CONCLUSIONS 205

LIST OF FIGURES

FIGURE		Page
2.1	Geographic distribution of the *C. variipennis* complex	32
2.2	A PCA and phylogenetic tree of the *C. variipennis* complex	33
2.3	The IBD for each species	34
2.4	Loci under selection	35
2.5	A haplotype network of the *C. variipennis* complex	36
3.1	The larval habitat of *C. grandensis*	69
4.1	Proposed suppression and replacement approaches for *Culicoides* population	202
4.2	Systematic steps to development of next-generation control methods	203
4.3	Side by side comparison of *Culicoides* eggs with other Diptera	204
5.1	A comparison of trees constructed using SNP data and mitochondrial data	211

LIST OF TABLES

TABLE		Page
2.1	Collection site information for chapter II	30
2.2	Mean pairwise *FST* within and between species	31
2.3	Mean percent divergence (p-distance) within and between species	31
3.1	A summary of the Nei's genetic distance of the *C. variipennis* complex	147
5.1	The known bionomic information of the species within *C.* (*Monoculicoides*)	206

CHAPTER I

INTRODUCTION: *CULICOIDES* BITING MIDGES & EVOLUTION

Species Delimitation

The way we separate organisms into species varies depending on the criteria being considered to make this assessment [1, 2], however, this delimitation can have far-reaching implications on governmental policies, pest and vector control, and biological conservation, as well as other fields of biology [3, 4]. Numerous species concepts have been proposed and refined over the years as new insights into the speciation process have emerged [5-8]. The issue is that species can arise in many different ways and the biology of some organisms is so unique that they are not always comparable. Still, there is a push for an all-encompassing set of rules of the delineation of species during any point in the speciation process [9, 10]. Contemporary species concepts have moved away from the rigidity of older theories and have instead adopted a more fluid and multifaceted approach to species delimitation. By examining evidence derived from morphological, ecological, and genetic data, we gain a better understanding of the evolutionary forces maintaining species boundaries in these organisms. There are, however, many biological processes, such as ecological or temporal variation in morphology, cryptic taxa, hybridization, and endosymbionts which can introduce uncertainty into any of these species delimitation tools as well as produce conflicting results between methods [11-14].

Distinct populations usually experience differing selection pressures, and this in turn can lead to morphological variation [15]. Morphological species identification can be easy for separating fully diverged taxa, but it can be challenging at the species level when only subtle differences exist. Naturally occurring morphological variation within a species is sometimes

described as evidence of different species, and this can take years of careful study to disentangle. The usefulness of this delimitation method is also limited when species are so morphologically similar that it takes expertise or specialized equipment to differentiate them [16]. Cryptic species are closely related organisms so similar that the boundaries between them are often unclear and can arise due to a recent speciation event, convergent evolution, or subtle adaptations [17]. Nonetheless, our inability to separate these species does not mean there is no reproductive isolation. When studying species, identifying ecological differences in habitat, food preference, or behavior can aid in their separation, as two species cannot occupy the exact same ecological niche without significant competition [18]. This may also provide insight into what reproductive isolation barriers are present within a study system [5]. Like morphological study, ecological variation within a single species can differ greatly, and so again, intensive studying may be required to properly identify separate species. The time and effort required to study and delimit species through morphological and ecological traits, and this is likely the reason molecular species identification has become so appealing [19-22].

Genetic sequences obtained from certain barcoding regions are used to group species based upon overall genetic similarity. The underlying principle of this method is the amount of genetic variation within a single species will be less than the variation between species [23]. This method of species delimitation has been used to detect cryptic species, link adults to their immature stages, and to identify native and introduced populations of a single invasive species. Molecular identification requires much less training and can offer a level of taxonomic resolution not possible with morphology alone. The databases used to store and compare these molecular data are also expanding: genetic sequences are currently available for approximately 250,000 to 300,000 species [24]. The use of molecular data in species delimitation has also led to the

conceptualization of new species concepts (e.g., Genetic Species Concept or the Gene and Gene Expression Species Concept). Molecular species identification, however, is still subject to over- or under-splitting species similar to the other delimitation methods [1]. The rate and level of divergence are not uniform for all organisms, and as such, setting a universal threshold of what amount of genetic divergence constitutes a species group appears to be unfeasible. Additionally, the amount of divergence within certain lineages does not necessarily align with the divergence of the genes being used in species delimitation, which may lead to discordance between delimitation methods or between genetic markers used for delimiting species [10].

Culicoides Biting Midges

Culicoides is a genus of blood-feeding flies that first evolved at least 99 million years ago [25, 26] and currently contains almost 1400 recognized species [27]. In this time, adaptations have arisen that have allowed this group to utilize a wide variety of habitats and hosts [28]. The immatures of most *Culicoides* species live in aquatic or semiaquatic habitats; some species have evolved to survive in highly specialized habitats such as rotting cacti, fungi, or highly saline pools [29]. The larvae are highly mobile and feed on organic debris or microorganisms, while the pupae spend most of their time buried in the larval habitat substrate with their respiratory organs protruding above the surface [29]. Like many other blood-feeding Diptera, adult female *Culicoides* require a vertebrate blood meal to develop a clutch of eggs, although many species are initially autogenous [30]. Most *Culicoides* species are considered either ornithophilic, mammalophilic, or generalist, with one notable exception wherein a few species take a secondary bloodmeal from engorged mosquitoes or blackflies [31]. In combination with these biological traits, *Culicoides* are separated into numerous subgenera and species groups based

mainly on phenetics [27]. The monophyly of most subgenera, however, has yet to be tested; determining the relationships within this genus will remain limited until a thorough cladistics analysis is conducted.

Because of the blood-feeding behavior of female *Culicoides*, some species vectors of many disease-causing pathogens in both managed and natural settings [32]. They are known to vector over 70 viruses, 16 protozoans, and 29 filarial worms, as well as produce a painful bite that can cause allergic reactions [33]. The most economically important of these pathogens are African horse sickness virus (AHSV), Akabane virus (AKAV), bovine ephemeral fever virus (BEFV), bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), Schmallenberg virus (SBV), and likely Vesicular stomatitis (VSV). Additionally, biting midges can achieve high population densities, increasing nuisance biting as well as transmission rates, and have a high dispersal capability which can facilitate the spread of these pathogens [34]. Over the past 20 years, *Culicoides* midges have been responsible for several major disease outbreaks on almost every continent [35-37], and as such, a better understanding is needed as to why some species are more competent vectors as well as the best means by which to control them [38, 39].

Culicoides midges utilize wind-mediated dispersal capabilities for both short and long-range dispersal[40, 41]. Accordingly, studies have shown a high level of connectivity between populations of biting midge species, even at great distances [42-44]. This high mobility is an important factor to consider in regards not only to disease transmission, but also in regards to the evolution of the taxa within the genus. In a species for which geographic isolation would be difficult, how then is speciation initiated? With the biological diversity that occurs within *Culicoides*, it would seem that ecological isolation could play a large role in the diversification

within this group. However, in lieu of geographic isolation, behavioral or other ecological adaptations would need to be in place to maintain these species boundaries [5].

Overview

Culicoides midges are important ecologically and economically as pathogen vectors. Additionally, the high level of diversity within this group offers an intriguing system by which to study evolution. In this dissertation, I investigated the population structure of the *C. variipennis* complex using SNP and mitochondrial data. The inferred structuring within the complex was informative as to the true number of species as well as the level of gene flow present between them. I also completed a monograph of the subgenus *C*. (*Monoculicoides*) which includes taxonomic reassessments, keys to the males and females of the Palearctic and Nearctic, redescriptions of the adults, distribution maps, and a cladistical analysis. I further described the pupae of Nearctic *C*. (*Monoculicoides*) and provided a key to species. Finally, I reviewed the feasibility of developing and utilizing a sterile insect, *Wolbachia*-based, or genetic control program for use against *Culicoides* biting midges.

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CHAPTER II

SPECIATION IN THE FACE OF LONG-RANGE DISPERSAL

Introduction

Speciation is a dynamic evolutionary process through which populations segregate into independently evolving lineages over time [1]. When gene flow is restricted between populations, the accumulation of genetic changes, through selection or local genetic drift, may lead to genetic differentiation and potentially reproductive isolation [2-5]. This restriction of gene flow occurs through either geographic or ecological isolation, though these are not mutually exclusive [6]. Thus, the level of gene flow between divergent populations is a contributing factor to the rate of speciation, as well as to the spatial level at which it occurs [7].

Geographic isolation reduces migration between populations, and thus, life-history traits influencing dispersal ability can drastically influence the level of gene flow among populations (*e.g.* [8, 9]. Species with low dispersal ability are particularly likely to exhibit highly differentiated populations resulting in the evolution of cryptic species over a limited spatial scale [10]. In contrast, species with high dispersal abilities are likely to maintain gene flow between populations, therefore a process outside of geographic isolation is needed to initiate the speciation process in these instances [11-13]. Two such mechanisms for this are ecological and behavioral isolation whereby sympatric populations occupy distinct ecological niches [14, 15]. The difference between these populations, such as habitat type, differences in mating times, or sexual selection reduce the frequency of interbreeding and this can lead to genetic divergence [2, 16]. Ecological and behavioral isolation can drive lineage divergence through selection, and subsequent pre-zygotic isolation can further increase divergence through reinforcement, accentuating the speciation

process [2]. However, with an increase in specialization, fragmented distributions of either habitat or host may further reduce gene flow between populations [17-19].

Each reproductive isolation mechanism can lead to similar morphological adaptations and genomic signatures [20, 21], and this can make it challenging to interpret what factors contributed to the speciation event. While the most accurate assumptions about species delimitation are derived from a multifaceted approach [22, 23], gene flow is directly tied to the fate of incipient species. By using a population genetic approach to study microevolution and incipient speciation, we can identify independent lineages and measure the introgression between them to better understand the evolutionary processes underlying species divergence. Species delimitation is especially important when working with organisms responsible for pathogen transmission, as misidentifications will lead to inaccurate vector competency and surveillance data. *Culicoides* Latreille (Diptera: Ceratopogonidae) biting midges are responsible for the transmission of many disease-causing agents worldwide [24, 25], including bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV). These viruses can cause severe symptoms and death in wild and domestic ungulates and are responsible for substantial economic losses globally [26, 27].

In North America, one of the main BTV and EHDV vectors is *Culicoides sonorensis* Wirth and Jones, which belongs to the *C. variipennis* species complex. When originally described, this group consisted of five subspecies [28], but it is currently considered to be composed of three distinct species (*C. occidentalis* Wirth and Jones, *C. sonorensis*, and *C. variipennis* (Coquillet)) [29]. Despite the current taxonomic arrangement, species identification remains difficult due to very subtle adult morphological differences and genetic similarity. Additionally, cryptic species could make vector incrimination and species distribution records potentially unreliable. Measuring genetic divergence between species and populations can be useful in vector biology as vectorial

capacity and host association become increasingly variable with increased genetic distance [30, 31]. Population genetic studies of *Culicoides* species in Europe, Africa, and Australia have consistently revealed frequent gene flow between populations, even at continental scales [32-35]. Their high dispersal ability, likely wind-mediated [36, 37], decreases the likelihood of geographic isolation between populations of *Culicoides* spp. Under laboratory conditions, at least two species within the *C. variipennis* complex have been shown to hybridize [38], and while *C. occidentalis* and *C. variipennis* are not known to be competent vectors, both species occur sympatrically with *C. sonorensis* [28]. This lack of post-zygotic reproductive isolation, coupled with a high dispersal ability and numerous sympatric populations, makes this species complex an intriguing system in which to study speciation and may also provide insights into the evolutionary mechanisms responsible for vector competence in this group.

Here, we evaluated the geographic connectivity within and among the species of the *C. variipennis* complex by assessing the level of gene flow within and across populations. We used a high-throughput ddRadSeq protocol to analyze 206 individuals collected from 17 sites throughout the United States and Canada. We first estimated the overall genetic similarity and population structure among these samples to determine distinct lineages within the species complex. We then estimated the level of gene flow between the inferred species, as well as uncovered hybridization between sympatric species. As previous attempts to separate these species using common barcoding genes have been inconclusive, we sequenced a region of COI to compare to the inferred SNP identifications. One species, was found to have two distinct geographic haplogroups, while three other species shared a single haplogroup. Additionally, we assessed the potential drivers of divergence in this species complex by assessing loci under selection for each species, as well as discuss the potential mechanisms controlling reproductive isolation.

Materials and Methods

Sample collection and sequencing

Culicoides midges were collected from 17 sites across the United States and Canada (Table 1). Specimens were collected either as pupae and reared to adulthood, or as adults using CDC light traps baited with CO_2 and UV light (Bioquip 2836BQ). Individuals morphologically assigned to the *C. variipennis* complex were sorted out from the by-catch and stored in 95% ethanol at -80 °C. Total DNA was extracted from individuals (females only) using a Puregene extraction protocol (Gentra Systems, Inc., D-5500A) with the addition of glycogen (ThermoFisher, R0561) to increase yields. The DNA quality was checked using gel electrophoresis and DNA concentration was measured using a Qubit 3.0 fluorometer and a Qubit dsDNA HS assay kit (Invitrogen, Q33230). A total of 300-400 ng of DNA per sample was sent to Floragenex, Inc. for library preparation using the protocol from Truong et al. (2012). DNA was digested using the restriction enzymes *MseI* and *PstI*. After PCR amplification, the samples in each plate were pooled and sequenced on a lane of single-end 100bp sequencing on a HiSeq4000 at the University of Oregon Genomics Facility, Eugene, OR.

Raw sequence filtering and processing

Raw sequence quality was first assessed using FastQC v.0.11.9 and MultiQC v.1.7 [39, 40], and then reads were filtered and processed using Stacks v.2.3 [41]. Reads with a phred score below 25 were removed as well as individuals with a >75.0% missing data. Next, reads were aligned to the *C. sonorensis* genome [42] (Accession: PRJEB19938) using the Burrows-Wheeler Aligner (BWA-mem) [43]. Finally, aligned reads were run through the reference-based pipeline of Stacks, with filtering parameters set to keep SNPs occurring in at least half of the sampling locations and at least 50% of individuals within those sites [44]. The minimum allele frequency was set to 0.05 to

protect against potential sequencing errors [45], and only the first SNP per locus was kept to minimize linkage disequilibrium between SNPs from influencing population structure and phylogenetic analyses. All subsequent file reformatting was done with PGDSpider v.2.1.1.5 [46].

Clustering Analysis

Population structure in the overall dataset was evaluated using fastSTRUCTURE v.1.04, with Structure_threader utilized to parallelize distinct runs of K [47, 48]. Initially, samples were grouped by location and no species data were pre-assigned to the individuals. To estimate the most likely number of genetic clusters in the dataset (K), the analysis was run for values of K ranging from 1 to 17 (*i.e.*, number of sites sampled). The best value was selected using the *chooseK.py* function from the fastSTRUCTURE package and plots were created by Distruct v.2.3 (http://distruct2.popgen.org). The clustering of individuals into the distinct genetic groups were also visualized using a principal component analysis (PCA) and a discriminant analysis of principal components (DAPC). The most likely number of genetic groups was inferred by the *find.clusters* algorithm for the PCA and the optimal number of principal components to inform the DAPC was defined using the function *optim.a.score*. Both were performed in R [49] through the *adegenet* package [50].

Any individual with more than 25% of their loci grouping with a second cluster was marked as a hybrid and removed from the phylogenetic analysis. Maximum likelihood phylogeny among individuals was run using RAxML v.8.2.12 [51]. An acquisition bias correction was applied to the likelihood calculations as alignments were solely composed of SNPs, with each invariant site removed through Phrynomics (https://github.com/bbanbury/phrynomics) [52]. The GTR+G nucleotide substitution model was used for each search. A rapid bootstrap analysis and search for the best-scoring maximum likelihood tree was executed using the extended majority rule-based

bootstopping criterion to achieve a sufficient number of bootstrap replicates [53]. Additionally, to cross-validate our results, a second phylogeny was inferred in W-IQ-Tree version 1.6.12 [54], using the TVM+F+G4 substitution model determined by ModelFinder [55, 56]. Branch support was calculated using 1000 ultrafast bootstraps [57] and Shimodaira–Hasegawa like approximate likelihood-ratio test (SH-aRLT) [57, 58].

As each of these clustering methods consistently supported five genetically distinct clusters, we generated a SNP dataset with individuals assigned to both a sampling location and a cluster ("all-species" dataset) as well as four species-specific datasets. SNPs were generated from the raw reads following the processing methods above except the filtering parameters were increased to only include SNP that occurred in at least 75% of the populations and at least half of the individuals within those populations. Genetic diversity estimates (F_{IS}, H_E, and H_O), population differentiation (pairwise F_{ST}), and isolation-by-distance (IBD) were calculated for each SNP dataset using Genepop v.4.7.0 [59]. Geographic distances were calculated as Euclidean distances among localities.

Mitochondrial Sequencing and haplotype network

Mitochondrial DNA haplotypes were obtained from a subset of 67 individuals from the five genetic clusters. PCR reactions were performed using a Taq-Pro COMPLETE kit (Denville Scientific, CB4065-4) targeting a partial region of the COI gene with the Lep50 primer set from Folmer et al. (1994) and the thermocycler profile from Herbert et al. (2003). PCR products were cleaned using an EXOSAP-IT kit (ThermoFisher, 78201.1.ML) and prepared for sequencing using a BigDye Terminator v.3.1 Cycle Sequencer Kit (Applied Biosystems, 4337454). Sanger sequencing was done using an Applied Biosystems 3500 Genetic Analyzer. Chromatograms were cleaned and aligned using the program Geneious v.9.1 [60].

A haplotype network analysis was conducted using the 67 COI sequences obtained in this study combined with 218 *C. variipennis* complex sequences previously collected (M. Hopken unpublished data). Sequences were aligned in MEGA v.10.1.8 [61] and trimmed to 546 bp to ensure all sequences contained identical lengths. A median-joining analysis was performed using NETWORK v.5.0.1.0 [62]. Specimens collected in this study were assigned a color based on the results from the SNP clustering analyses while the remaining samples were left unassigned. All individuals were used to calculate the mean uncorrected *p*-divergence between and within the different groupings inferred from the haplotype network using MEGA.

Outlier loci detection

The "all-species" and species-specific datasets analyzed in Genepop were also run through Bayescan v.2.1 to identify loci under divergent selection [63]. Parameters of the Markov chain Monte Carlo algorithm were set to 20 pilot runs of 5000 iterations. Afterward, a burn-in of 50,000 iterations followed by 50,000 iterations were used for estimation with a thinning interval of 10. Jeffrey's scale was used to interpret selection per loci [64]. Loci with a log10 value > 0.5 are considered to have "substantial" evidence of selection and those with a log10 value > 1.0 have "strong" evidence of selection. To identify loci under selection across clusters another new SNP dataset was generated by filtering to include only those occurring in all five clusters and 75% of the individuals within each cluster. The nucleotide sequences for each locus found to be under selection were submitted for an alignment search in the InsectBase and Flybase databases [65, 66].

Results

In total, 271 individuals were subjected to the ddRADseq procedure and yielded an average of 2.08 million reads per individual. During the initial filtering, 36 individuals had a phred score of

less than 25 and were removed from the dataset. Additionally, 29 individuals were found to have more than 75% missing data and were therefore removed. The final dataset included 206 individuals from 17 sites and contained 3612 SNPs. The population structure inferred by fastSTRUCTURE that best explains the data is K = 5. At K = 5, most individuals (86%) were unambiguously assigned to one group (98-100% assignment score; Fig. 2.1). Consistent with these results, the PCA and DAPC grouped these individuals into five main clusters (Figs. 2.2a). The main difference being that the PCA further segregated one cluster (blue, Fig. 2.2a) into two separate groups; east and west of the Sierra Nevada mountain range. Further support for the same five clusters was found in the maximum likelihood trees, with a high level of support from each approximation method (Figs. 2.2b).

The geographic distributions of four of these clusters closely align with the distributions of four of the five subspecies described in Wirth & Jones (1957) (Fig. 2.1), suggesting these morphological descriptions accurately denoted species-level taxa within the *C. variipennis* complex. Further phylogenetic and morphological study is needed to confirm the validity of these species groupings; however, for the remainder of the manuscript we will refer to each cluster by a species name. *Culicoides occidentalis* located in Western North America, *C. sonorensis* in the Western and Southern U.S., *C. albertensis* in the Midwest U.S. and Canada to Ontario, *C. variipennis* in the Eastern U.S. and Ontario, and a fifth genetic group suggesting the occurrence of an additional, undescribed cryptic species in San Diego, CA. Notably, eight of the 17 sites had more than one species in sympatry, and one site had three species. At four sites, seven individuals were assigned to two genetic groups with an assignment score of ~50% for three individuals (scores = 45, 47 and 41%) and of ~25% for four individuals (scores = 34, 31, 25 and 24%), which suggests the occurrence of putative F1 or other types of hybrids (e.g. F2 or backcrosses),

respectively. Interestingly, these hybrids were from three different species parings (*C. sonorensis* X *C. occidentalis*; *C. sonorensis* X *C. variipennis*; and *C. albertensis* X *C. variipennis*). These hybrid individuals also stood out using the PCA analysis, as they segregated between their parental clusters (Fig. 2.2a), as well as at the base of each parental branch in the phylogenetic tree. In addition to these hybrids, 20 individuals had a secondary assignment score between 3% to 21%, signifying potential introgression between those pairings.

The samples were then rearranged by species, rather than collection site, and stricter filtering parameters were applied. This dataset contained 566 SNPs from 199 individuals (hybrids were excluded) and was used to calculate the species-level summary statistics as well as determine the loci under selection. The mean F_{ST} between the five inferred clusters was 0.7147 (0.6541-0.7470), roughly 9 times higher than the mean F_{ST} between the populations (i.e., localities) within each cluster (see below; Tables 2 & S1). The overall dataset was further split into five datasets for species-level population statistics. These datasets contained 22 individuals of *C. albertensis* from four populations (3423 SNPs), 36 individuals of *C. occidentalis* from four populations (2714 SNPs), 97 individuals of *C. sonorensis* from seven populations (2357 SNPs), and 29 individuals of *C. variipennis* from four populations (2960 SNPs). The expected and observed heterozygosity, F_{IS}, and number of private alleles for each species are reported in Table S2. No species level dataset was created for the San Diego species as only one locality was examined.

When examining each species individually, *C. albertensis,* had no evidence of population structure (K = 1), and had low genetic differentiation among populations (mean F_{ST} = 0.054) (Fig. 2.3a; Table 2.2). Although there does seem to be a pattern of isolation by distance, this was found to not be significant in this species (P = 0.238). The low number of populations sampled potentially limits our statistical power for this correlation. The results obtained for *C. occidentalis* showed

much more divergence compared to the other species, with populations being strongly differentiated from each other (mean F_{ST} = 0.411) (Table 2.2). Additionally, fastSTRUCTURE suggests that each population of *C. occidentalis* is a distinct genetic entity (K = 4) clustering by location (Fig. 2.3b). While no IBD was found (P = 0.489), there seems to be a considerable amount of geographic isolation among populations of this species, with pairwise F_{ST} values ranging from 0.14 to 0.70. Low genetic differentiation among populations was found for *C. sonorensis* (mean F_{ST} = 0.029), despite a slight, but significant IBD in this species (P = 0.039) (Fig. 2.3c; Table 2.2). For this reason, the individuals from Colorado were combined into a single population, as were the individuals from Kansas. A fastSTRUCTURE analysis suggested the occurrence of population structure in *C. sonorensis* (K = 2), with some individuals from Kansas belonging to a distinct group. The combined Kansas populations were not divergent from any other *C. sonorensis* population (Table S3). Populations of *C. variipennis* exhibited no evidence of population structure (K = 1) or of isolation by distance (P = 0.587) (Fig. 2.3d). Consistently, almost no genetic differentiation was found among populations of this species (mean F_{ST} = 0.026) (Table 2).

We identified three outlier loci within the *C. variipennis* complex and an additional 23 species-specific loci: two in *C. albertensis*, seven in *C. occidentalis*, 11 in *C. sonorensis*, and two in *C. variipennis*. Each of these loci had a log10 Bayes factor value over 1 and six had values above 2, corresponding to a 95% and 99% confidence interval, respectively (Fig. 2.4). Searches of InsectBase were used to assign putative functional annotations (most of which were provided by Nayduch et al. (2014), with orthologous dipteran genes subsequently found using Flybase (Table S4). Roughly 75% of the loci were matched to transcription data, and all but one associated with a dipteran orthologous gene. None of the loci found to have significant evidence of selection were shared across the different species, suggesting that each is under its own set of selective pressures.

Based on the COI gene, four distinct groupings were identified with strong genetic divergence between groups (*p*-distance = 2.99-3.30%) and little divergence within groups (*p*-distance = 0.25-0.86%; Fig. 2.5; Table 2.3). Consistent with the SNP datasets, the California population of *C. occidentalis* was separated from the rest of its range. The mean percent divergence between the two *C. occidentalis* groups (2.99%) was similar to its divergence from the other species (3.01-3.30%). The San Diego population clusters as a distinct group, with a similar level of divergence from the other species (3.01-3.03%). Interestingly, *C. albertensis*, *C. sonorensis*, and *C. variipennis* were not separated from each other, and in some cases, *C. albertensis* and *C. variipennis* shared identical haplotypes (Fig. 2.5). Furthermore, these three species exhibit a mean percent divergence between individuals (0.80%) similar to the divergence observed among individuals within the other clusters (Table 2.3). Other than the grouping of *C. occidentalis* in California, there was no other geographic clustering observed.

Discussion

Our study provides valuable insights into the population genetics of the *C. variipennis* species complex and highlights the presence of potential cryptic species. For most of the species examined, minimal genetic divergence was observed across populations, suggesting the maintenance of gene flow even over large geographic distances. The only exception was *C. occidentalis*, which showed a high level of geographic isolation, as well as two distinct genetic clusters. We confirmed that mitochondrial data is not reliable to properly differentiate three out of five species, due to the lack of segregation between the mitochondrial haplotypes of *C. albertensis*, *C. sonorensis*, and *C. variipennis*. This stands in stark contrast to their clear differentiation and high level of divergence inferred from the SNP data. Though a substantial amount of divergence exists between all five

species, hybridization and introgression are present at low levels in sympatry suggesting that post-zygotic isolation barriers have not evolved in this group. Thus, pre-zygotic isolation through either ecological or behavioral segregation is likely responsible for divergence within this complex. With a considerable amount of overlap between some species (Fig. 2.1), each sympatric population is potentially experiencing a set of unique selective pressures to maintain species boundaries.

Species delimitation and dispersal capabilities within the C. variipennis complex

The high degree of genetic differentiation between clusters inferred by the SNP data supports the current species groupings of the *C. variipennis* complex (*C. occidentalis*, *C. sonorensis*, and *C. variipennis*), as well as raising *C. albertensis* and a cryptic species in San Diego, California to species status. Little to no IBD or structure was found within populations of *C. albertensis*, *C. sonorensis*, and *C. variipennis* (Fig. 2.3a,c,d). The number of populations inferred by fastSTRUCTURE for *C. sonorensis* was K=2; however, a mean pairwise F_{ST} of 0.0287 suggests that a high amount of gene flow still exists between all populations. This could also be an artifact of the propensity of delta K inferring two populations [67] or from a high level of relatedness among individuals from KS.

Interestingly, although no IBD was found in *C. occidentalis*, each location of this species clustered as a distinct population. The lack of IBD is therefore not indicative of a single, genetically homogeneous population, but rather stems from high levels of divergence between populations regardless of their geographic distances. In this species, the strong genetic divergence between the population from California and the other populations observed in the SNP data was consistently uncovered in the mtDNA (4.0% divergent, Table 2.3, Fig. 2.5). It is possible that this may represent a further cryptic species with a dispersal barrier created by the Sierra-Nevada mountain range. Patchiness of the larval habitat of *C. occidentalis* could also create isolation between populations

as well as reduce the number of individuals within each population. A small population size with little to no immigration would allow for a strong effect from drift [68]. While the populations of *C. occidentalis* outside of California were less diverged from one other, the lowest pairwise F_{ST} values between these populations were still greater than the highest pairwise values observed for any other species, consistent with the findings of Holbrook et al. (2000) (Table 2.2). Interestingly, at one of the three loci found to be under selection with the complex (seipin, Table S4), all populations of *C. occidentalis* and *C. albertensis* were fixed for a single allele, whereas the other three other species were fixed for the other alternative allele. This SNP was determined to be synonymous and therefore unlikely to be the direct target of selection; however, it may be linked to a region of the genome that is.

Similar to other species of *Culicoides* [32, 35, 69, 70], high values of the inbreeding coefficient were observed in all species investigated in this study. Although these previous studies have suggested that the observed high inbreeding coefficient values are an artifact from a large number of null alleles, the consistent reporting of these findings across various species using several types of molecular markers lends support to the hypothesis that high inbreeding has a biological origin. High levels of inbreeding and heterozygote deficiencies are common among mosquitoes [71-73], even when using markers with a low level of null alleles [74, 75]. Goubert *et al*. (2016) considered the typical *Aedes albopictus* population as "a network of interconnected breeding sites, each with a high level of inbreeding". In this study, although we cannot rule out that the presence of null alleles and we acknowledge that a weak Wahlund effect can contribute to the level of inbreeding, our results strongly suggested that some aspects of the reproductive biology of *Culicoides* induce inbreeding within populations.

mtDNA and nuclear discordance

Culicoides albertensis, *C. sonorensis*, and *C. variipennis* have a considerable amount of genome-wide differentiation (Fig. 2.1); however, there was no clear differentiation of the COI gene (Fig. 2.5). In fact, several individuals of *C. albertensis* and *C. variipennis* shared identical haplotypes. Multiple studies have shown a high degree of genetic similarity in mtDNA between *C. sonorensis* and *C. variipennis* [76-78], though it was proposed that this was due to misidentifications. As all of the individuals included in our mitochondrial haplotype analysis from the current study were identified to species using the SNP data, this lack of mitochondrial separation has an underlying biological source. Ongoing hybridization with "leaky" pre-zygotic isolation, or a semipermeable species boundaries, has been shown to produce mitochondrial introgression without detectable nuclear DNA introgression [79, 80]. This is likely due to the fact the mitochondrial genome is independent of the nuclear genome and thus unlinked to the genes contributing to reproductive isolation [81]. This does not appear to be the case throughout the entire complex though as hybridization was also found between *C. sonorensis* and *C. occidentalis* and the mtDNA from these two species was highly divergent.

In addition to the convergence of a single haplogroup by three species, *C. occidentalis* was found to have two distinct haplogroups based on geography (Fig. 2.5). The mean percent divergence between *C. occidentalis* from California (CABL) and *C. occidentalis* from the other collection sites (BC-NV-UT) was equal to the divergence between the other species in the complex (Table 3). This high level of differentiation within *C. occidentalis* could be due to geographic isolation alone; however, endosymbionts have also been shown to significantly increase mitochondrial diversity in the presence of geographic structure [82, 83]. Naturally occurring endosymbionts have been found in *Culicoides* midges, including *C. sonorensis* [84, 85], and recently, a *Cardinium* sp. was linked to mitochondrial divergence of *C. imicola* [86]. Further

screening is needed to determine the diversity and abundance of endosymbionts infecting *Culicoides* midges, though the possibility remains that these could be playing a role in the phylogeographical structure of *C. occidentalis*.

Hybridization and reproduction isolation

Under laboratory conditions, mating between *C. sonorensis* and *C. occidentalis* can produce viable offspring for at least six generations, though the hatch rate of the progeny is dependent on the species of the mother [38]. A cross of female *C. sonorensis* and male C. *occidentalis* only yields a 7% hatch rate whereas the reciprocal cross yields a 75% hatch rate. This asymmetrical hybrid viability is likely caused by cytonuclear incompatibility [87, 88], though endosymbionts have also been shown to cause reproductive incompatibility [89]. Upon secondary contact of closely related species, and in the absence of post-zygotic reproductive isolation, the production of unfit hybrids can induce the rapid evolution of premating barriers [2, 90-92]. In most populations however, *C. sonorensis* females are unlikely to come across *C. occidentalis* males due to differences in mating behavior. Conversely, *C. occidentalis* females do come into contact with *C. sonorensis* males, who do not appear to have mate discrimination [93], and will likely attempt to mate with these heterospecific females. As there are demographic disparities (population size and structure) between these two species, as well as viable offspring produced from this cross, rampant hybridization and asymmetric introgression would be detrimental to *C. occidentalis* [94]. Strong selection against hybridization can maintain species boundaries, but as two of the ten *C. occidentalis* collected from Borax Lake in California (CABL) were F1 hybrids, another mechanism, potentially ecological or behavioral isolation, appears to be limiting directional introgression from *C. sonorensis*.

Culicoides occidentalis females lay their eggs in highly saline environments (up to 88.0 parts per thousand (ppt)) [95], and *C. sonorensis* eggs will not hatch in water with salinity over 20.0 ppt [96]. Ecological exclusion via the larval habitat is present in this system, but alone would only limit introgression if the survival rate of the hybrids were reduced. *Culicoides occidentalis* mate at the larval habitat while *C. sonorensis* mates at or near a host [29, 97], and this difference in mating behavior may be a more likely mechanism by which the detrimental effects of hybridization are diminished. Most *C. occidentalis* females will mate at the larval habitat, but if this does not happen, they may be mated by *C. sonorensis* while feeding at the host. As *C. occidentalis* females return to the high saline pools to lay their eggs, these hybrid individuals would have an increased chance of backcrossing with the *C. occidentalis* lineage. While only two *C. occidentalis* x *C. sonorensis* hybrids were tested in this study, both had *C. occidentalis* mothers, providing some evidence that this is the scenario taking place in nature.

Impact on vector competency and future work

The *C. variipennis* complex is one of many vector groups in which species delimitation can be challenging [98-102]; however, species identification is an integral part of vector surveillance. The species status of these group members has implications for vector surveillance, as any ambiguity in identification will lead to unreliable data. For example, while *C. albertensis* and *C. sonorensis* occur in sympatry, only *C. sonorensis* is a reported as a vector species [103]. The addition of the non-competent vector species when conducting serological surveys could lead to a severe underestimation of the infection rate within the vector species. As BTV and EHDV are expanding northward into eastern Canada [104], it has been suggested that the dispersal of *C. sonorensis* to new areas could be to blame for this incursion [77]. Specimens assigned to *C. sonorensis* by Jewiss-Gaines et al. (2017) were included in the present study and cluster instead with *C.*

albertensis ("ON", Fig. 2.1). Thus, there are likely alternative reasons for this expansion, including an unidentified vector species outside of the *C. variipennis* complex. Accurate species-level delimitation within this complex is sorely needed for proper vector surveillance. Additionally, elucidating the evolutionary history of these groups can lead to a better understanding of how some species become highly competent vectors while closely related taxa are not. The detection of hybridization within a vector species may be evidence of recent speciation, but it also highlights a potential path of introgression for genes controlling vector competency [105, 106].

Conclusion

Our study shows that using a population genomic approach to studying sibling species can identify both species-level divergence as well as fine-scale genetic structuring. Tracing the level of gene flow within and between these species enables the detection of geographic isolation, hybridization, and cryptic species to offer a more accurate depiction of the current species dynamics. Radiation within the *C. variipennis* complex occurred despite the long-range dispersal capabilities of biting midges as well as hybridization between sympatric species. Because of this, we believe that behavioral and ecological isolation may have shaped evolution within this group or is at least maintaining the existing species boundaries. Significant geographic isolation was only found between populations of *C. occidentalis*, but more work is needed to determine if the lack of gene flow between California and the other populations represents an incipient speciation event. Delimiting the species in the *C. variipennis* complex will not only aid in vector surveillance efforts, but continued study of the speciation of closely related vector and non-vector species could produce valuable evolutionary insights into vector competency.

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Tables

Table 2.1. Collection site information and numbers of individuals retained for the SNP analyses.

Country	State/Provence	Lat	Long	Collection date	Collection method	N	Abbreviation
Canada	British Columbia	49.3065	-119.6323	5/7/2019	Pupal rearing	5	BC
USA	California	39.0245	-122.8515	8/14/2018	Pupal rearing	12	CACL
USA	California	38.9811	-122.6731	8/14/2018	Pupal rearing	9	CABL
USA	California	32.5522	-117.0628	11/7/2014	Light trap	15	CASD
USA	Idaho	43.7065	-116.4236	8/19/2014	Light trap	14	ID
USA	Nevada	40.0521	-118.4681	7/29/2013	Light trap	17	NV
USA	Arizona	34.5792	-112.4258	7/21/2010	Light trap	17	AZ
USA	Utah	40.7844	-112.1090	9/10/2018	Light trap	16	UT
USA	South Dakota	43.7438	-101.9509	8/6/2018	Light trap	10	SD
USA	Colorado	40.6560	-104.9878	8/8/2019	Light trap	15	COFC
USA	Colorado	39.0546	-108.5170	7/16/2013	Light trap	7	COME
USA	Kansas	38.8793	-98.4481	9/25/2018	Pupal rearing	16	KSLI
USA	Kansas	39.2234	-96.5906	7/17/2018	Light trap	18	KSMA
USA	Texas	29.9515	-99.6010	7/29/2017	Light trap	8	TX
Canada	Ontario	43.2167	-79.9500	7/5/2013	Light trap	8	ON
USA	South Carolina	34.3080	-81.7550	7/23/2014	Light trap	16	SC
USA	Florida	30.4782	−84.6401	8/27/2018	Light trap	3	FL

Table 2.2. Mean pairwise F_{ST} within and between species. The between species F_{ST} values (below diagonal) were calculated using 566 SNPs and the within-species values (on diagonal) is the mean F_{ST} calculated from individual species-specific datasets (see Table S3).

Species	*C. albertensis*	*C. occidentalis*	*C. sonorensis*	*C. variipennis*
C. albertensis	0.055 *(-0.009–0.116)*	-	-	-
C. occidentalis	0.707	0.411 *(0.143 – 0.704)*	-	-
C. sonorensis	0.709	0.730	0.029 *(0.006 – 0.069)*	-
C. variipennis	0.654	0.747	0.730	0.026 *(-0.006 – 0.045)*
San Diego pop.	0.714	0.719	0.706	0.734

Table 2.3. Mean percent divergence (p-distance) within and between species clusters based on the COI gene (ranges listed in parentheses). Based on overall similarity, *C. occidentalis* was split into two groups (CABL; and BC-NV-UT) and *C. albertensis*, *C. sonorensis*, and *C. variipennis* were grouped into a single clade (alb-son-var).

Clade	occ (CABL)	occ (BC-NV-UT)	San Diego pop.	alb-son-var
occ (CABL)	0.48 (0.00 – 0.73)	-	-	-
occ (BC-NV-UT)	3.99 (3.20 – 5.49)	0.86 (0.00 – 1.65)	-	-
San Diego pop.	3.01 (2.38 – 4.21)	3.66 (2.75 – 4.76)	0.25 (0.00 – 0.66)	-
alb-son-var	3.30 (2.75 – 5.12)	3.76 (3.30 – 6.04)	3.03 (2.38 – 4.21)	0.80 (0.00 – 2.74)

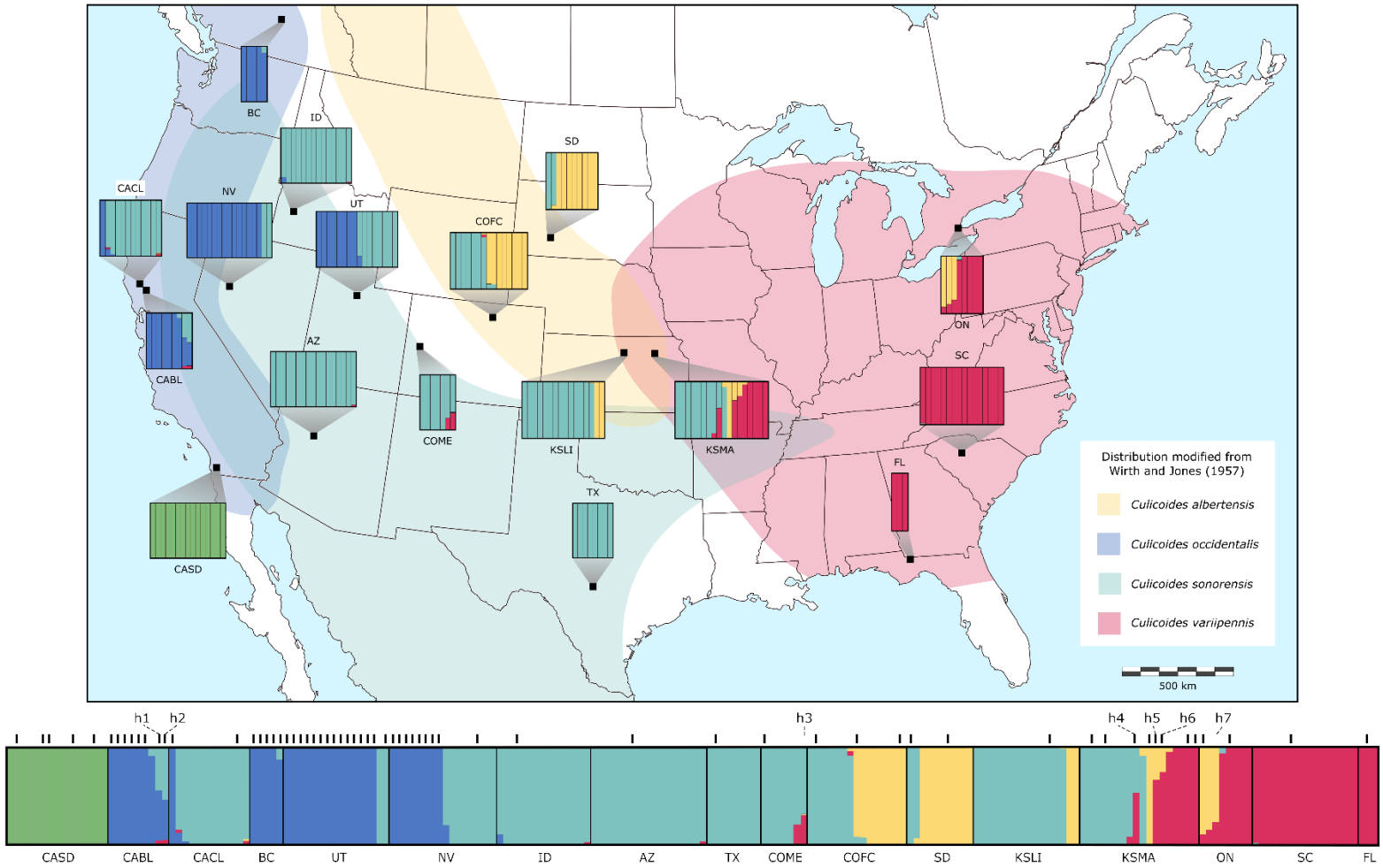

Figure 2.1. Geographic distribution and structure plots for each collection site (black squares) overlaid on the historical distribution of the species described in Wirth and Jones 1957. The fastSTRUCTURE results are for 206 individuals inferred by 3612 SNPs and assuming five populations (K=5). The vertical bars within each collection site represents an individual, with each color representing a cluster. The putative species identity of each clusters are as follows: *Culicoides occidentalis* (blue), *C. sonorensis* (teal), *C. albertensis* (yellow), *C. variipennis* (red), and an unidentified population in San Diego, CA (CASD) (green). The black bars above structure plot indicates an individual for which the COI gene was also sequenced. The individuals inferred to be hybrids are labeled h1-7.

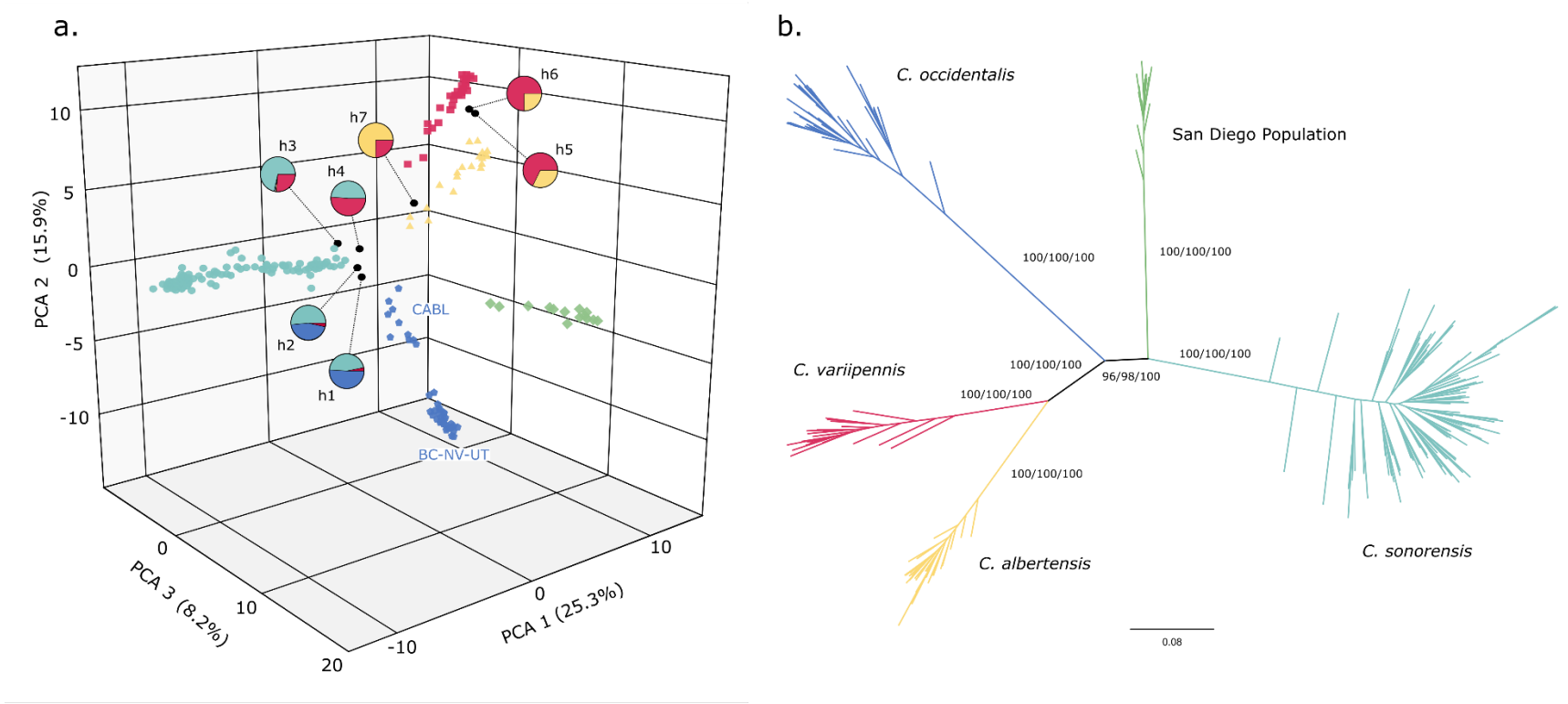

Figure 2.2. (a) A 3D representation of the principal Component Analysis (PCA) of all individuals included in the study. Each color represents the cluster inferred from the structure analysis; *C. albertensis* (yellow), *C. occidentalis* (blue), *C. sonorensis* (teal), *C. variipennis* (red), and the unidentified San Diego population (green). Hybrids (h1–h7) are designated with a black circle and their inferred parental ancestry is depicted with pie graphs. The geographic locations of the two *C. occidentalis* clusters are labeled next to each grouping (see table 1 for abbreviation). **(b)** Unrooted maximum likelihood phylogenetic tree based on 199 individuals inferred from 3612 SNPs (the hybrids were removed here but are included in Fig. S3.). Clade colors represent the clusters inferred from the structure analysis; *C. albertensis* (yellow), *C. occidentalis* (blue), *C. sonorensis* (teal), *C. variipennis* (red), and the unidentified San Diego population (green). Support values written on the branches: rapid bootstrap (%) / SH-aLRT support (%) / ultrafast bootstrap support (%). For clarity, the values within each cluster are not shown.

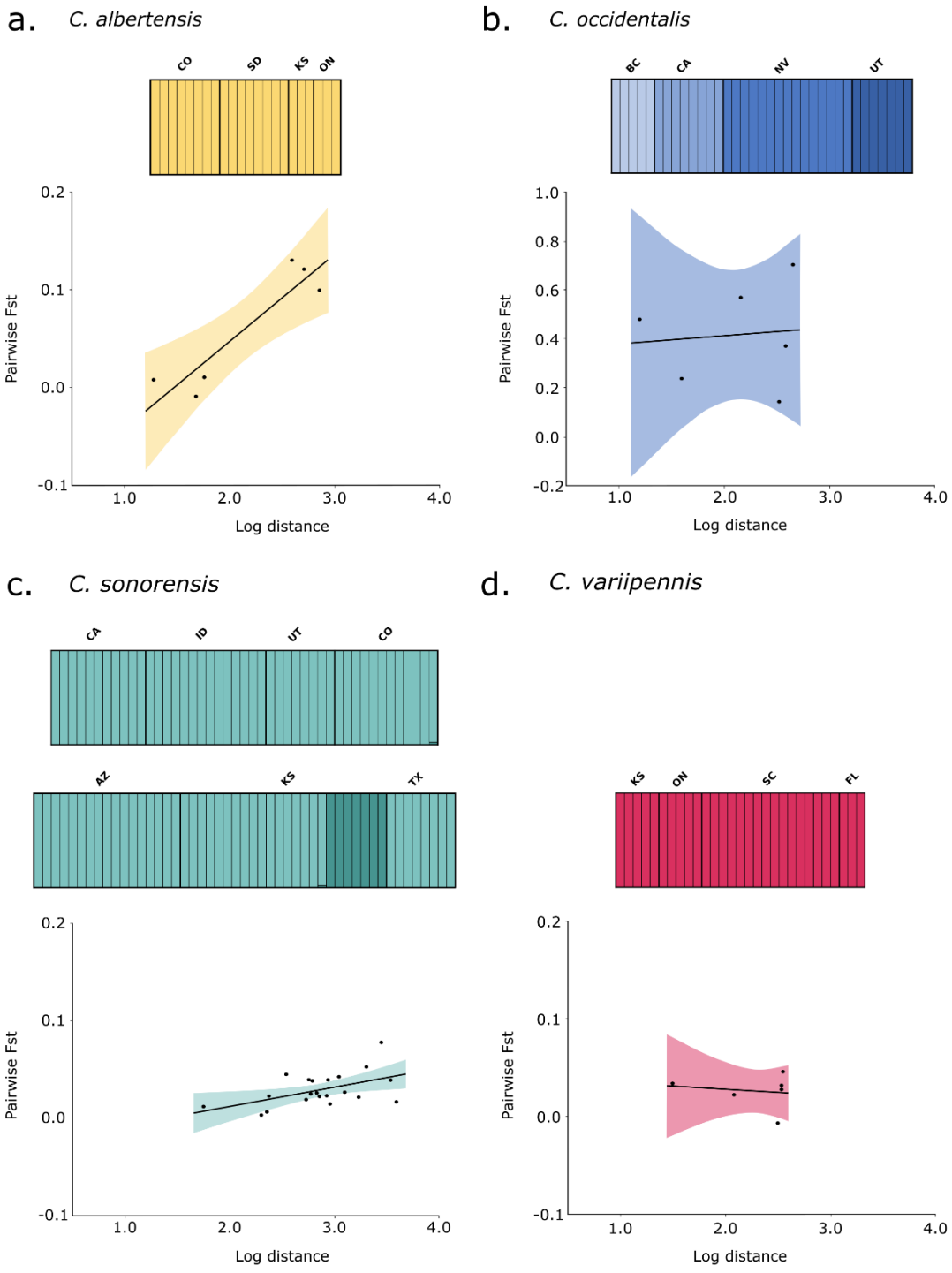

Figure 2.3. For each species, an independent SNP dataset was used to calculate the best K using fastSTRUCTURE v.1.04 with the inferred clusters denoted by varying shades. The IBD (shown as pairwise F_{ST} by log geographic distance) for each species were calculated in Genepop v.4.7.0. The individuals from San Diego, CA are not included here as they were only found in a single population.

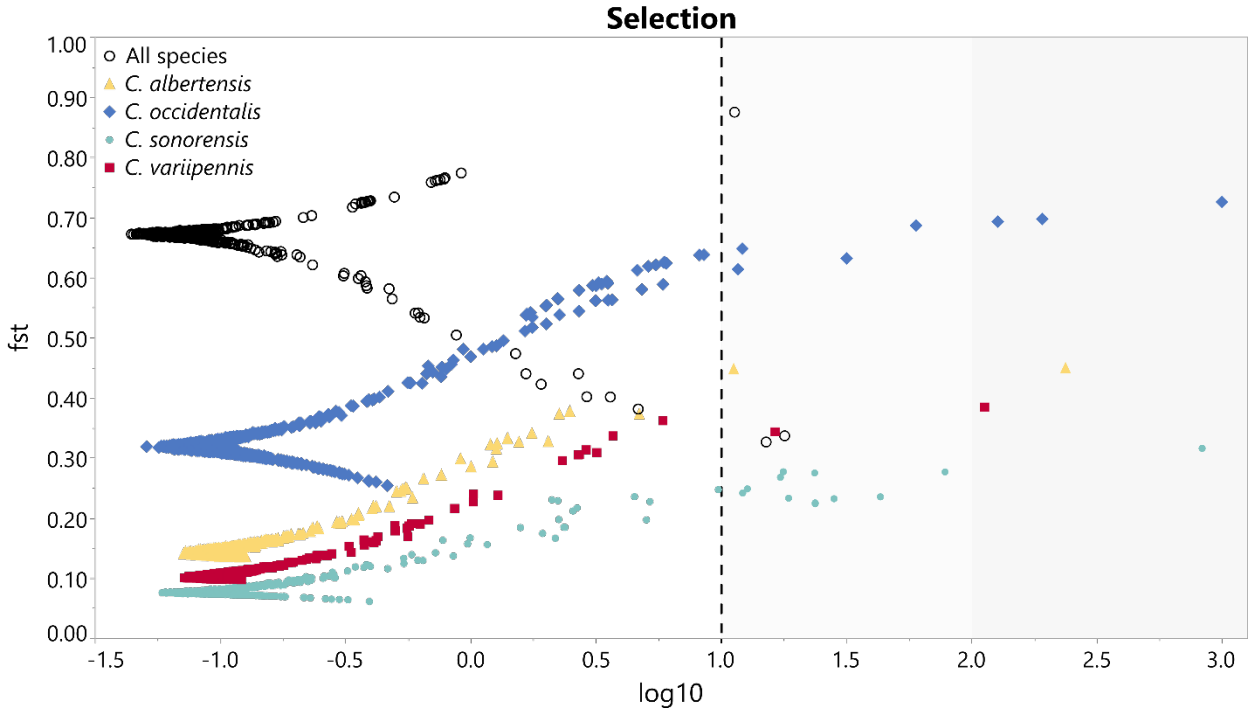

Figure 2.4. Loci under selection. Individual loci from the "all-species" dataset (566 SNPs) and the species-specific datasets are plotted against their corresponding log10 values. A log10 over 1.0 is considered to have high support (95% CI) for being under selection with a log10 value over 2.0 corresponding 99% CI for being under selection. The individuals from San Diego, CA do not have a species-specific dataset as they were only found in a single population, however, they were still included in the "all species" analysis.

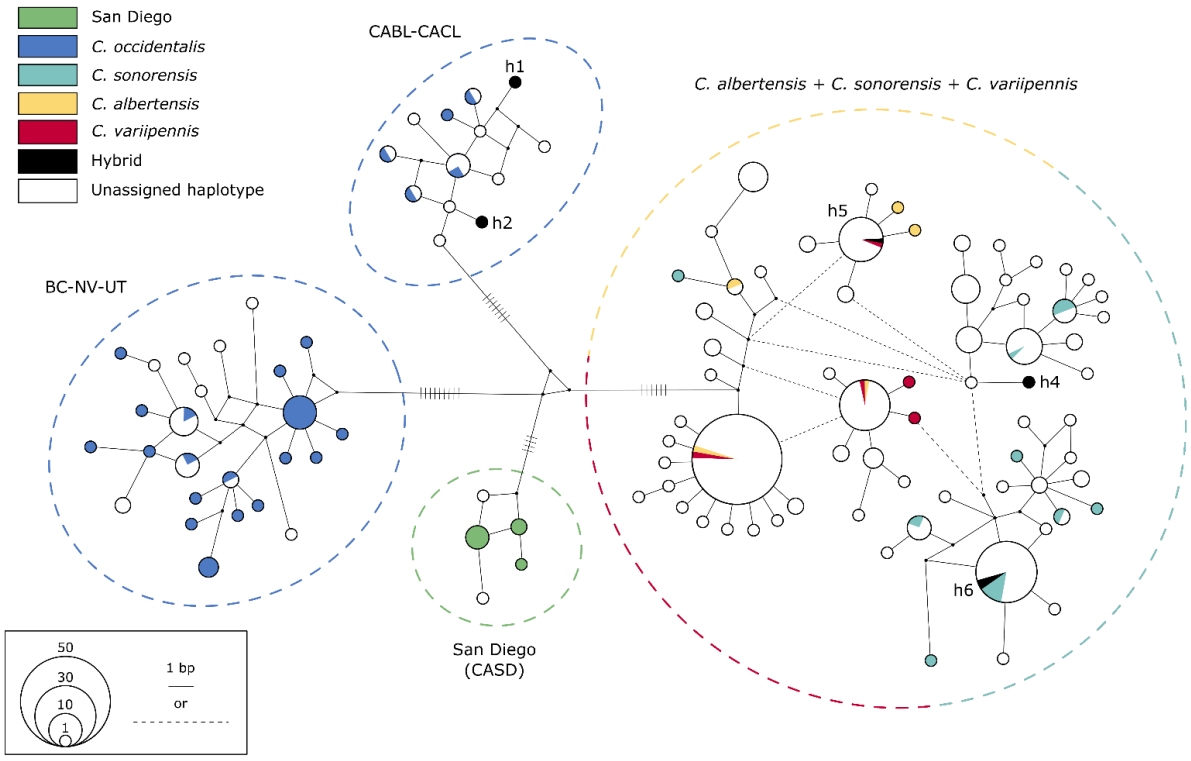

Figure 2.5. A haplotype network inferred by a median-joining method, using 285 mitochondrial (mt) DNA sequences of the *C. variipennis* complex from 27 states in the U.S. as well as British Columbia and Ontario, Canada. The size of each circle represents the frequencies of the haplotype. The 67 sequences obtained in the present study, see figure 1, are colored according the clusters assigned from the structure analysis. The four main groups of haplotypes are demarcated by ellipses (see main text).

CHAPTER III

A TAXONOMIC REVISION OF THE SUBGENUS *C*. (*MONOCULICOUDES*)

Introduction

The genus *Culicoides*, also known as biting midges, are in the family Ceratopogonidae. Currently, there are 1399 extant species and 52 fossil species placed into 33 subgenera and 38 species groups, with another 136 species unplaced [1]. Apart from being ecologically important as pathogen vectors [2], this group has a high level of diversity, offering a plethora of systems in which to study evolution. In addition, Ceratopogonidae has one of the most complete fossil records within insects dating back nearly 125 million years [3, 4]. The ability to look back in time and have a reference for the plesiomorphic conditions within a group is invaluable to cladistical analysis, but for now, taxonomic uncertainties and little support for the overall arrangement of the genus have limited our ability to draw robust phylogenetic conclusions. Only some species of *C*. (*Avaritia*) [5], all *C*. (*Monoculicoides*) *[6]*, and *C*. (*Groganomyia*) (only one extant species) [7] have even been proposed as monophyletic with support from cladistical analyses. Until the monophyly of the other subgenera are tested and more synapomorphies are found, we will not be able to interpret the morphological, biological, and ecological features of *Culicoides*.

Culicoides belongs to an unresolved group with 11 species of *Paradasyhelea* + one species of *Washingtonhelea* that together make up the tribe Culicoidini; the sister group to all higher Ceratopogonidae [8]. These genera also represent some of the more basal lineages of Ceratopogonidae. Recently, Szadziewski *et al*. (2019) proposed that a new subgenus C. (*Groganomyia*) represents the most basal lineage of Culicoidini and that this tribe is paraphyletic. While the relationship between C. (*Groganomyia*) and the other genera within

Culicoidini remains unclear, this subgenus could at the very least be the sister to all other *Culicoides*. The confirmation of this subgenus as the basal group within the genus would aid in the politzerization of at least some characters states. It also provides a testable hypothesis for future cladistical analyses.

To date, there have been few cladistical analyses published for any group within *Culicoides*, and the current arrangement of the genus is exclusively phenetic. In order to further test the validity of the subgenus *C*. (*Monoculicoides*), we provide a taxonomic revision with redescriptions of the adults, keys to species, as well as bionomic and taxonomic discussions for each species. We also critically assess the historical and new evidence of speciation within the variipennis complex.

LOCATION OF TYPE MATERIAL

This list is not complete as not all authors listed where their material was deposited and the type material for some species could not be located. Additionally, only a limited amount of material was available for loan during the course of this study due to the Covid-19 pandemic. Acronyms for museums are those proposed by Borkent (2020).

BGBM—Botanischer Garten und Botanisches Museum Berlin-Dahlem Freie Universität Berlin; Berlin, Germany.
CNC—Canadian National Collection of Insects; Ontario, Canada.
EIHIU—Entomological Institute of the Hokkaido Imperial University; Hokkaido, Japan
HNHM—Zoological Dept., Hungarian Natural History Museum; Budapest, Hungary.
IMBC—Medical Insect Collection, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences; Beijing, People's Republic of China.
NHMUK—The Natural History Museum; London, United Kingdom.
NMPC—National Museum of Natural History; Prague, Czech Republic.
NMSA—Natal Museum; Natal, South Africa.
NYSM—New York State Museum; New York, USA.
NZCI—National Zoological Collections of India; West Bengal, India
SNMG—Staatliches Museum für Naturkunde; Danzig, Poland

USNM—National Museum of Natural History; Washington, D.C., USA.
ZIN—Russian Academy of Sciences, Zoological Institute; St. Petersburg, Russia.
ZMHB—Museum für Naturkunde der Humboldt Universität zu Berlin; Berlin, Germany.

Materials and Methods

Adults specimens were collected using baited and un-baited CDC light traps. Pupae were collected using the methods of Borkent (2014). Slide mounted, alcohol, and pinned material of various species of the subgenus *C.* (*Monoculicoides*) were borrowed from the following collections: CNC, NHMUK, NYSM, SNMG, USNM, and ZIN. Freshly-collected and museum specimens stored in alcohol were slide-mounted in Canada balsam according to Borkent and Spinelli (2007) and observed with a Nikon Alphashot-2 YS2 compound microscope and a Leica S6D dissecting microscope. Terms and abbreviations follow those used by the Manual of Afrotropical Diptera Volume 2 [9]. Descriptions follow an anterior to posterior and dorsal to ventral organization. Features present in all *Culicoides* are not repeated here.

The species list below reflects the results of the current dissertation. Any nomenclatural actions or descriptions of taxa in this dissertation are not considered to be validly published under the rules of the ICZN, and they are not intended to be part of the permanent, public, scientific record.

Subgenus *MONOCULICOIDES* Khalaf

MONOCULICOIDES Khalaf, 1954: 39 (as subgenus of *Culicoides*). Type species: *Ceratopogon nubeculosus* Meigen, by original designation.
STIGMOCULICOIDES Isaev, 1988: 15 (as subgenus of *Culicoides*). Type species: *Culicoides stigma* (Meigen), by original designation.

albertensis Wirth and Jones, 1957. **New status.**
australis Wirth and Jones, 1957. **New status.**
combinothecus Yu and Liu, in Yu et al., 1986.

cornutus de Meillon, 1937 .
digitalis Remm, 1973.
xinghaiensis Yu, 1982. **New synonym.**
expallens Remm, 1973.
erkaensis Yu and Yang, in Yu 1988. **New synonym.**
grandensis Grogan and Phillips, 2008.
heiheensis Li, Zhang and Liu, 2011.
aihuiensis Wu, Jiao and Liu, 2019. **New synonym.**
helveticus Callot, Kremer and Deduit, 1962.
homotomus Kieffer, 1922.
osakensis Iwata 1935.
denmeadi Causey 1938.
buhetoensis Takahashi 1941.
obtusus, Chatterjee, Brahma & Hazra 2020. **New synonym.**
lochmocola Yu, Ayiken et Chen, 2016.
longicollis Glukhova, 1971.
paradoxus Yu & Liu 1990.
longlinensis Yu, 1982.
mullensi n. sp. Shults and Borkent 2021.
nanpingensis Yu and Song, in Yu et al., 1986.
nubeculosus (Meigen), 1830.
puncticollis Goetghebuer 1912.
punctaticollis Goetghebuer 1920.
occidentalis Wirth and Jones, 1957.
pachynonus n. sp. Shults and Borkent 2021.
parroti Kieffer, 1922.
puncticollis (Becker), 1903.
algecirensis (Strobl) 1900.
impressus Kieffer 1918.
distigma Kieffer 1922.
donatieni Kieffer 1922.
sciniphes Kieffer 1925.
bipunctatus Vimmer 1932.
tripunctatus Vimmer 1932.
wenigi Vimmer 1932.
flavitarsis Vimmer 1932.
griseovittatus Vimmer 1932.
luteosignatus Vimmer 1932.
vavrai Vimmer 1932.
riethi Kieffer, 1914.
cordatus Kieffer 1921.
crassiforceps Kieffer 1924.
gigas Root & Hoffman 1937.
shemanchuki Grogan and Lysyk, 2015.
sonorensis Wirth and Jones, 1957.

stigma (Meigen), 1818.
kiefferi Goetghebuer 1910.
cordiformitarsis Carter 1916.
unimaculatus Goetghebuer 1920.
stigmoides Callot, Kremer & Deduit 1962.
taonanensis Ren, Wang and Liu, 2006.
variipennis (Coquillett), 1901.

ADULT DESCRIPTION: Medium to relatively large sized species, usually yellowish brown or dark brown in color. Eyes broadly separated with a pair of distinct frontal tubercles present on the frontal carina; sensilla coeloconica absent on flagellomeres 9-13 and with female antennal ration between 0.75-1.00. Females of most species with 10-16 well developed mandibular teeth; palps variable from extremely wide with deep sensory pit to narrow without a defined sensory pit. Scutum grey, punctuated with small dark black spots or uniformly black or brown. Wings pale, with or without darker pigmented patterning, but always with black pigmentation complexly covering second radial cell cell; species with pigmented patterning also with distinct dark spot on wing just posterior to arculus and M3+CuA vein. Legs usually with light banding patterns; hind tibial comb with 4-7 spines, with the first two spines longer than the flowing. Male genitalia with parameres fused medially; bifurcated aedeagus, with or without spicules; dorsal root of gonocoxite large; gonostylus tapering gradually for basal half or tapering distally throughout; caudal margin of the epandrium straight, with medial notch, or with distinct posterior projection medial to base of apicolateral process; apicolateral process generally well developed. Females with a single spermatheca, always with wide opening at duct, shape variable; spermathecal ring present in some species.

DIAGNOSIS: *Male*: The only group of species of *Culicoides* with enlarged frontal tubercles on head, parameres broadly fused mediobasally, and with a bifurcate aedeagus. Those species with a wing pattern are unique in the genus in having a well-developed dark spot on the wing just

posterior to the arculus. *Female*: The only group of species of *Culicoides* with enlarged frontal tubercles on head and a single spermatheca with a large spermathecal duct opening. Those species with a wing pattern are unique in the genus in having a well-developed dark spot on the wing just posterior to the arculus. *Pupa*: The only group of species of *Culicoides* with dark banding on the apex of the pedicel and the base of the respiratory organ, with the respiratory organ elongate and slender, with a dark apex, and the midlength portion bearing scales, and terminal processes extending posterolaterally [10]. *Larva*: The only group of species of *Culicoides* with a greatly enlarged epipharyngeal complex [11-15]. *Egg***:** The only group of species of *Culicoides* in which the *ansulae elongate* are present and arranged in a random pattern [12].

Taxonomic Discussion

The designation of *C*. (*Monoculicoides*) in Khalaf (1954) was based on the presence of only one spermatheca and the second radial cell of the wing being fully pigmented. Originally, the subgenus included 21 species divided into four species groups: the *fulvithorax* group, the *guttifer* group, the *crepuscularis* group, and the *nubeculosus* group. Everything not in the nubeculosus group was subsequently removed from the subgenus. The *nubeculosus* group was originally coined by Edwards (1939) to group the British fauna of *C. nubeculosus*, *C. parroti*, *C. puncticollis*, *C. riethi*, and *C. stigma* based on the male and female genitalia. To these species, Khalaf (1954) included *C. variipennis* and *C. hegneri*. Later, Wirth and Jones (1957) removed *C. hegneri* from *C*. (*Monoculicoides*) and added *C. homotomus* (as *C. denmeadi*), *C. riethi* (as *C. gigas*). In addition to the characters listed previously, Wirth and Jones (1957) note several characters that we consider to be diagnostic or synapomorphic for some species in this subgenus; a lower antennal ratio (AR), sensilla coeloconica only on flagellomere 1-8, large frontal tubercles

and a large body size. Glick (1990) provided a description for the adults of *C.* (*Monoculicoides*), and other than listing the parameres as being fused basely, we generally agree with his assessment. Shults (2015) and Shults and Borkent (2018) report dark pigmentation around the pedicle of the pupae of Nearactic *C.* (*Monoculicoides*) and propose this as a synapomorphy for the group. Kettle and Lawson (1952) and Gutsevich and Glukhova (1970) notes that the larvae of *C.* (*Monoculicoides*) have a massive epipharyngeal complex, a synapomorphy for the group.

Grogan and Lysyk (2015) proposed the *nubeculosus-stigma* species complex which included four species of Nearctic *C.* (*Monoculicoides*): *C. grandensis*, *C. shemanchuki*, *C. riethi*, and *C. stigma*. Whereas this group would denote the North American species not in the *variipennis* complex, there is little morphological evidence for this grouping. We instead propose a revised *stigma* group which includes, *C. stigma*, *C. helveticus*, *C. parroti*, *C. combinothecus*, *C. nanpingensis*, and *C. digitalis*. Diagnostic characters for this group are the presence of a spermathecal ring, amorphous spermatheca or spermatheca with finger-like extension, lack of wing pattern (present in *C. digitalis*), and an AR equal to 1.0 (not present in *C. digitalis*). All of these features appear to be derived within *C.* (*Monoculicoides*). *Culicoides digitalis* has retained the pleomorphic states of patterning on the wing and an AR well below 1.0, but this provides evidence that this species is the sister taxa to the rest of the *stigma* group.

Key to Males of *Culicoides* (*Monoculicoides*) of the Nearctic Region

1) Wing with dark spot on wing just posterior to arculus and M_3+CuA vein ....some species of ***C.* (*Monoculicoides*)** (6)

– Wing without dark spot just posterior to arculus .... **other species of *Culicoides* including other *C. (Monoculicoides)*** (2)

2) Wing with pattern of pigmentation, or if unpatterned, microtrichia abundant, without a solitary dark spot completely covering second radial cell .....**other species of *Culicoides***

- Wing pale, reduced microtrichia, with solitary dark spot over second radial cell ....**other species of *Culicoides*, including *C.* (*Monoculicoides*)** (3)

3) Parameres fused medially; aedeagus bifurcate ..... ***C.* (*Monoculicoides*)** (4)
– Parameres separated or fused basally; aedeagus not bifurcated…….
.....**other species of *Culicoides***
4) Scutum black; paramere tips widely separated; epandrium with large posterior projection medial to base of apicolateral process ..... ***C. stigma*** (Holarctic, in Nearctic: Alberta)
– Scutum brown; paramere tips touching or nearly touching; epandrium without large posterior projection or only slight projection medial to base of apicolateral process..... 5
5) Epandrium with lateral margins parallel, only slightly tapering posteriorly, with slight posterior projection medial to base of apicolateral process ..... ***C. grandensis*** (Utah)
– Epandrium strongly tapering from base, without posterior projection medial to base of apicolateral process.....***C. shemanchuki*** (Alberta to North Dakota)
6) Black spots on scutum obscure; posterior margin of segment 9 with medial cleft .....
..... ***C. riethi*** (Holarctic, in Nearctic: Alaska to Manitoba south to Nebraska)
– Black spots on scutum prominent; posterior margin of segment 9 straight 7
7) With spicules covering more than half of the aedeagus; gonostylus gradually tapering to distal end..... *C.* ***sonorensis*** (Most of North America west of the Mississippi river)
– Aedeagus with spicules only at tip or absent, gonostylus tapering sharply at midpoint 8
8) Aedeagus with spicules only at tip, immature habitat alkaline .....
.....***C. albertensis*** (Alberta and the great plains of the USA)
– Aedeagus without spicules..... 9
9) Sensilla coeloconica present on flagellomere 1 and six or more of flagellomeres 2-8 .....
..... ***C. australis*** (Southeastern USA)
– Sensilla coeloconica present on flagellomere 1 and three to four of flagellomeres 1-8 ... 10
10) Distributed in the eastern US and Canada ..... ***C. variipennis***
– Distribution in the western US and Canada 11
11) Scutum grey with black dots, wing length less than 1.7 mm .....
.....***C. occidentalis*** (Western USA and CAN)
– Scutum light brown with black dots set in dark brown stripes, wing length greater than 1.7 mm ***C. mullensi*** (California)

Key to Females of *Culicoides* (*Monoculicoides*) of the Nearctic

1) Wing with dark spot on wing just posterior to arculus and M_3+CuA vein of wing.....
.....some species of ***C.* (*Monoculicoides*)** (6)
– Wing without dark spot just posterior to arculus.....
..... **other species of *Culicoides* including other *C. (Monoculicoides)*** (2)
2) Wing with pattern of pigmentation, or if unpatterned, microtrichia abundant, without a solitary dark spot completely covering second radial cell.....**other species of *Culicoides***
- Wing pale, reduced microtrichia, with solitary dark spot over second radial cell .....
..... **other species of *Culicoides*, including *C.* (*Monoculicoides*)** (3 sp. below)
3) Eyes separate (width of 1–4 ommatidia); with sensilla coeloconica present on flagellomere 1 and on three or more of flagellomeres 1-8; one spermatheca with duct opening wide …….
..... ***C.* (*Monoculicoides*)** (4)
– Eyes separate (width of 1-2) or touching; with sensilla coeloconica on flagellomere 1 and one or more of flagellomeres 2–13; 1-3 spermatheca but if one spermatheca then with duct opening narrow .....**other species of *Culicoides***

4) Scutum uniformly brown or black 5
– Scutum light brown or grey, with black dots 7
5) With at least 6 mandibular teeth; scutum black; spermatheca with finger–like extension, with spermathecal ring ..... ***C. stigma*** (Holarctic, in Nearctic: Alberta)
– Without mandibular teeth; scutum brown; spermatheca without extension or ring 6
6) Wing length less than 1.5 mm, scutum light brown; spermatheca short, cylindrical .....
..... ***C. grandensis*** (Utah)
– Wing length greater than 1.5 mm, scutum dark brown; spermatheca ovoid .....
..... ***C. shemanchuki*** (Alberta to North Dakota)
7) Black spots on scutum not prominent, spermatheca ovoid .....
..... ***C. riethi*** (Holarctic, in Nearctic: Alaska to Manitoba south to Nebraska)
– Black spots on scutum prominent, spermatheca "C–shaped" 8
8) Sensilla coeloconica present on flagellomere 1 and six or more of flagellomeres 1-8 9
– Sensilla coeloconica present on flagellomere 1 and three or four of flagellomeres 1-8..... 10
9) Distributed in southern Alberta and Saskatchewan south to Kansas..... ***C. albertensis***
– Distributed from Oklahoma to Virginia south to Texas and Florida ..... ***C. australis***
10) Third palpal segment with small pit, palp ratio 3.0 - 3.3 (narrow).....
..... ***C. variipennis*** (Eastern USA and CAN)
– Third palpal segment with large pit, palp ratio 1.86 - 2.5 (wide) 11
11) Wing length < 1.6 mm; 7-11 plural setae on abdominal segment 1; immature habitat high in organic material ..... ***C. sonorensis*** (Most of North America west of the Mississippi river.)
– Wing length > 1.6 mm; >13 plural setae on abdominal segment 1; immature habitat saline 12
11) Wing length > 1.9 mml; with brown vittae on scutum; >20 plural setae on abdominal segment 1 ..... ***C. mullensi*** (known only from California)
– Wing length 1.6 - 1.9 mm; 14-19 plural setae on abdominal segment 1; without brown vittae on scutum..... ***C. occidentalis*** (Western USA and CAN)

Key to Males of *Culicoides* (*Monoculicoides*) of the Palaearctic

1) Wing with dark spot on wing just posterior to arculus and M_3+CuA vein .....
.....some species of ***C.* (*Monoculicoides*)** (7)
– Wing without dark spot just posterior to arculus .....
..... **other species of *Culicoides* including other *C. (Monoculicoides)*** (2)
2) Wing with pattern of pigmentation, or if unpatterned, microtrichia abundant, without a solitary dark spot completely covering second radial cell.....**other species of *Culicoides***
- Wing pale, reduced microtrichia, with solitary dark spot over second radial cell .....
.....**other species of *Culicoides*, including *C.* (*Monoculicoides*)** (four sp. below)
3) Parameres fused medially, bifurcate aedeagus ..... ***C.* (*Monoculicoides*) (**4)
– Parameres separated or fused basally; aedeagus not furcated ....**other species of *Culicoides***
4) Epandrium without large posterior projection medial to base of apicolateral process, paramere wide medially with apices widely separated..... 5
– Epandrium with posterior projection medial to base of apicolateral process 6
5) Scutum grey with numerous overlapping black dots distally; eyes bare .....
..... ***C. parroti*** (Europe and North Africa)
– Scutum unknown; eyes with fine pubescence ..... ***C. combinothecus*** (China)

6) Aedeagus triangular; base of parameres stout .....
.....***C. stigma*** (Holarctic, in Palaearctic: primarily Europe and Russia)
– Aedeagus with apical portion cylindrical; base of paramere thin .....
.....***C. helveticus*** (Eastern Europe and Russia)
7) Pattern of scutum known 8
– Pattern on scutum unknown (various species from China) 13
8) Scutum uniformly brown..... 9
– Scutum grey or light brown with black dots 10
9) Paramere tips elongate, aedeagus cylindrical ..... ***C. expallens*** (Mongolia)
– Paramere tips not elongate, aedeagus triangular..... ***C. digitalis*** (Mongolia)
10) Aedeagus cylindrical, scutum with prominent spots 11
– Aedeagus entirely triangular, scutum with diffused spots 12
11) Gonostylus tapering sharply at midpoint.. ***C. nubeculosus*** (primarily Europe and Russia)
– Gonostylus tapering gradually to apex .. ***C. longicollis*** (Eastern Europe and Western Asia)
12) Posterior margin of abdominal segment 9 with large, well-defined medial cleft.....
..... ***C. riethi*** (Holarctic, in Palaearctic: broadly distributed)
– Posterior margin of abdominal segment 9 with small, undefined medial cleft .....
.....*C.* ***puncticollis*** (Europe, North Africa, and the Middle East)
13) Paramere width greater than length 14
– Paramere width less than length 15
14) Gonostylus tapering sharply at midpoint; posterior margin of abdominal segment 9 with large, well-defined medial cleft ..... ***C. heiheensis*** (China)
– Gonostylus gradually tapering to distal end; posterior margin of abdominal segment 9 slightly concave ..... ***C. longlinensis*** (China)
15) Base of apicolateral process narrow; epandrium with large posterior projection medial to base of apicolateral process; apicolateral process long..... ***C. lochmocola*** (China)
– Base of apicolateral process wide; epandrium without large posterior projection medial to base of apicolateral process; apicolateral process greatly reduced ..... ***C. taonanensis*** (China)

Key to Females of *Culicoides* (*Monoculicoides*) of the Palaearctic

1) Wing with dark spot on wing just posterior to arculus and M_3+CuA vein of wing.....
.....some species of ***C.* (*Monoculicoides*)** (8)
– Wing without dark spot just posterior to arculus.....
..... **other species of *Culicoides* including other *C. (Monoculicoides)*** (2)
2) Wing with pattern of pigmentation, or if unpatterned, microtrichia abundant, without a solitary dark spot completely covering second radial cell.....**other species of *Culicoides***
- Wing pale, reduced microtrichia, with solitary dark spot over second radial cell .....
.....**other species of *Culicoides*, including some *C.* (*Monoculicoides*)** (four sp. below)
3) Eyes separated (length of 2–4 ommatidia); with sensilla coeloconica absent on flagellomeres 9-13; one spermatheca with duct opening wide ..... ***C.* (*Monoculicoides*)** (4)
– Eyes separate (width of 1-2) or touching; with sensilla coeloconica on flagellomere 1 and one or more of flagellomeres 2–13; 1-3 spermatheca but if one spermatheca then with duct opening narrow .....**other species of *Culicoides***

4) Spermatheca folded and amorphous, slightly folded onto itself 5
– Spermatheca spherical or with finger–like extension 6
5) Scutum grey with lateral black dots and wholly black medially; eyes bare .....
..... ***C. parroti*** (Europe and North Africa)
– Scutum unknown; eyes with fine pubescence ..... ***C. combinothecus*** (China)
6) Spermatheca spherical, without finger–like extension ..... ***C. nanpingensis*** (China)
– Spermatheca with finger–like extension 7
7) No pit on third palpal segment; scutum dark brown .....
.....***C. helveticus*** (Eastern Europe and Russia)
– Small pit on third palpal segment, scutum black .....
.....***C. stigma*** (Holarctic, in Palaearctic: primarily Europe and Russia)
8) Pattern of scutum known 9
– Pattern on scutum unknown (two species from China) 14
9) Scutum uniformly light brown 10
– Scutum grey to yellowish-brown, with black spots 11
10) Spermatheca with finger–like extension ..... ***C. digitalis*** (Mongolia)
– Spermatheca elongate and not highly sclerotized.....***C. expallens*** (Mongolia)
11) Black spots on scutum diffused; spermatheca straight 12
– Black spots on scutum prominent; spermatheca slightly curved 13
12) Spermatheca elongate ..... *C*. ***puncticollis*** (Europe, North Africa, and the Middle East)
– Spermatheca ovoid ..... ***C. riethi*** (Holarctic, in Palaearctic: broadly distributed)
13) Spermathecal duct swollen at opening of spermatheca, third palpal segment narrow .....
..... ***C. nubeculosus*** (primarily Europe and Russia)
– Spermathecal duct uniform at opening of spermatheca, third palpal segment wide .....
.....*C.* ***longicollis*** (Eastern Europe and Western Asia)
14) Spermatheca, ovoid ..... ***C. longlinensis*** (China)
– Spermatheca, spherical ..... ***C. taonanensis*** (China)

Species Descriptions

Culicoides albertensis Wirth and Jones

Culicoides (*Monoculicoides*) *variipennis albertensis* Wirth and Jones, 1957:17 (as subspecies). Type locality: Lethbridge, Alberta, Canada. Holotype ♀ pinned (CNCI), type number 356813, "Lethbridge Alta., 22 July 1955, J. A. Downes, 22/4/24"; Allotype ♂ slide (CNCI), same collection data; Paratypes 50 ♀♀ 35 slides, 15 pinned, 6 ♂♂ 3 slides, 3 pinned, Lethbridge, 19-22 July 1955, at light, J. A. Downes (CNCI, USNM); ♀ 1 slide, 17 June, 1955 (CNCI); ♀♀ 2 slides, 1 pinned, Fort MacLeod, [Canada], 22 July 1955, swept margin alkaline slough, J. A. Downes ♀♀ 2 slides, 1 pinned, ♂ 1 pinned (CNCI); ♀♀ 1 slide, 1 pinned, Brooks, [Canada] 18 July 1955, at light, J. A. Downes (CNCI).

Culicoides occidentalis albertensis: Downes 1978:63

Culicoides sonorensis albertensis: Holbrook *et a*l. 2000:70

Culicoides albertensis: This study. **NEW STATUS**

DIAGNOSIS: only species of *C*. (*Monoculicoides*) with diffused black spots on scutum, sensilla coeloconica present on six or more flagellomeres, and with spicules only at the tip of the aedeagus; female: only species of *C*. (*Monoculicoides*) with diffused black spots on scutum, sensilla coeloconica present on six or more flagellomeres, and with a C-shaped spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots; wings with faint pattern of pigmentation. **Abdomen:** abdominal segment 1 with 14-17 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process narrow at base, tapering to apex; fused parameres with long, stout base, apices narrowly separated; aedeagus triangular, with spicules only at apex; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 3-4 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 3-8; palpus with third segment wide with large pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 10-14; spermatheca ovoid, long, C-shaped, with spermathecal ring absent.

DISTRIBUTION: *Culicoides albertensis* is known from southern Alberta to Ontario south to Oklahoma and Ohio.

Bionomics

Adult Habitat and Seasonality

The range of *C. albertensis* cover most of the temperate grasslands, savannas & shrublands of North America, but this species has also been recorded in temperate broadleaf & mixed forests in the eastern USA and Canada [16]. Adult *C. albertensis* have been collected at light traps from late April to early October [17].

Immature Habitat

Larvae of *C. albertensis* are found in alkaline lakes and pools common in the Great Plains of North America [17]. Downes (1958) collected immatures of *C. albertensis* alongside *C. riethi* and *C. shemanchuki* from alkaline sloughs in southern Alberta.

Feeding

Biting mouthparts indicate that female *C. albertensis* likely feeds on vertebrate blood [18].

Mating behavior

Unknown.

Development

Unknown.

Vector status

Unknown.

Molecular data

Holbrook *et al*. (2000) mention that electrophoretic analyses were done on specimens collected in Warner, Alberta, (near the type locality of *C. albertensis*), and were determined to be *C. sonorensis*. Either the isozymes used in this study were unable to genetically isolate *C. albertensis* or the specimens analyzed were in fact *C. sonorensis*. Chapter II provides evidence of the species status of *C. albertensis* using SNP data indicating that it should be recognized as a distinct species. The level of genome-wide divergence between *C. albertensis* and the other members of the *C. variipennis* complex was equal to the level of divergence observed for *C. occidentalis*, *C. sonorensis*, and *C. variipennis*. Sequencing of the COI gene of *C. albertensis* also revealed that this species shared mitochondrial haplotypes with *C. sonorensis* and *C. variipennis*.

Taxonomic Discussion

In the original description of the subspecies *C. v. albertensis*, Wirth and Jones (1957) distinguished the females from that of C. *v. sonorensis* by their narrower third palpal segment, larger wing length, greater number of flagellomeres with sensilla celoconica, and a more diffused patterning of dots on the scutum. The males of both species are listed as having numerous spicules on the aedeagus. Downes (1978) found no evidence that *C. albertensis* should be considered anything more than a geographic variant of *C. occidentalis* and placed it there as a subspecies. Holbrook *et al*. (2000) reexamined much of the material identified as *C. albertensis* by Wirth & Jones (1957) and determined these specimens to be *C. sonorensis* based primarily on the presence of spicules on the aedeagus as well as some isozyme data (see above).

I believe that some of the confusion surrounding *C. albertensis* stems from a lack of clarification as to the number of spicules on the aedeagus. Wirth and Jones (1957) describes the aedeagus of *C. albertensis* as "bearing numerous fine ventral spines" and the aedeagus of male *C. sonorensis* as "bearing numerous, well-developed ventral spines on the main body." We believe that this very slight, yet significant, distinction of spicules on the main body was meant to differentiate this character as we have done in the current study; spicules only at the tip (*C. albertensis*) and spicules covering most of the aedeagus (*C. sonorensis*). Additionally, Blanton and Wirth (1979) listed differences in the wing length and palpal ratio between *C. albertensis* and *C. australis*; however, the range of both of these morphometrics overlaps greatly for these species [17].

Using isozyme data, researchers found only genetic evidence of three species within the *C. variipennis* complex, and so it was determined that any number of spicules on the aedeagus constituted a *C. sonorensis* male [19, 20]. We have examined the male paratypes of *C. albertensis* from Lethbridge, Canada and all have spicules only at the very tip of the aedeagus.

This is also the case for some of the specimens from Wyoming, Colorado, Kansas, and South Dakota. Additionally, we have found that the characters Wirth and Jones (1957) used to distinguish female *C. albertensis* also distinguish this species from *C. sonorensis*. These morphological differences, along with distinct larval habitats and a high genetic divergence, provide evidence that *C. albertensis* warrants full species status.

[21, 22]

Culicoides australis **Wirth and Jones**

Culicoides (*Monoculicoides*) *variipennis australis* Wirth and Jones, 1957:15 (as subspecies). Type locality: Baton Rouge, Louisiana, USA. Holotype ♀ pinned (USMN), type number 63248, 14 April, 1947 W. W. Wirth, at light; Allotype ♂ slide (USNM)), same location data, 6 May 1947; Paratypes ♀♀ 30 slides, 20 alcohol, ♂♂ 1 slides, 5 alcohol, 26 April 1947, over manure pile; ♀ 10 slides, ♂ 4 slides, 6 May 1947, at light; ♀ 10 pinned, ♂ 1 pinned, 11, 14, 26 April 1947, at light.
Culicoides occidentalis australis: Downes 1978:63.
Culicoides sonorensis australis: Holbrook *et a*l. 2000:70.
Culicoides australis: This study. **NEW STATUS**

DIAGNOSIS: Male: only species of *C.* (*Monoculicoides*) with prominent black spots and brown vittae on scutum, with sensilla coeloconica present on six or more flagellomeres, and aedeagus bare; female: only species of *C.* (*Monoculicoides*) with prominent black spots on scutum, sensilla coeloconica present on six or more flagellomeres, and with a C-shaped spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots; wings with faint pattern of pigmentation. **Abdomen:** abdominal segment 1 with 8-12 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process narrow at base, tapering to apex; fused parameres with long, stout base, apices narrowly separated; aedeagus triangular, without spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**:

eyes broadly separated by a distance equal to 3-4 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 2-8; palpus with third segment wide with large pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 10-14; spermatheca ovoid, long, C-shaped, with spermathecal ring absent.

DISTRIBUTION: *Culicoides australis* is known only in the USA from Kansas to Virginia, south to Texas and Florida.

Bionomics

Adult Habitat and Seasonality

Culicoides australis in the temperate forests and grasslands of the southeastern USA [16]. Of the specimens examined in Wirth and Jones (1957), *C. australis* have only been collected in March, April, and May, with peak emergence occurring in April [17, 22].

Immature Habitat

The larvae and pupae of *C. australis* have been collected and reared from saline pools and salt springs in Virginia, Missouri, and Louisiana [17, 23].

Feeding

In Missouri, numerous adult females found near the larval habitat were reported as landing on the exposed skin of humans, but did not attempt to feed [17]. The presence of finely serrate mandibles and retrorse teeth on the laciniae mean this species likely feeds on vertebrates [18].

Mating behavior

Zimmermann *et al*. (1982) describe the general characteristics of the male swarm of *C. variipennis* in Virginia such as size, shape, height, orientation, and flight path. It is; however, unclear which species Zimmermann *et al*. (1982) actually observed. Based on the morphology of the third palpal segments reported by these authors, the proportion of females collected from

these swarms was as follows: *C. australis* or *C. sonorensis* (60%) (referred to as *C. occidentalis*), *C. variipennis* (16%), and an intermediate form (24%). Holbrook *et al*. (2000) later identified all the material from this location as *C. variipennis*; however, we do not agree with this assessment. The third palpal segments of females that we have examined from this population align closer to the assessment made by Zimmermann *et al*. (1982), and these specimens also have a high number of sensilla celoconica. Additionally, these observations were made very close to a salt spring where many *C. australis* have been collected. For these reasons, we believe that the species Zimmermann *et al*. (1982) actually observed swarming was primarily *C. australis*.

Development

Unknown.

Vector status

Unknown.

Molecular data

Specimens of *C. australis* may have been included in the studies of Tabachnick (1992), Schmidtmann *et al*. (1988), and Holbrook *et al*. (2000), although none of these authors were able to differentiate this species using isozyme markers. No barcode is available yet for this species.

Taxonomic Discussion

In the original description of the subspecies of *C. australis*, Wirth and Jones (1957) note that this taxon was distinct from the rest of the *C. variipennis* complex based on having the highest number of flagellomeres bearing sensilla celoconica in both the male and female adults. This character state overlaps considerably with *C. albertensis*; however, *C. australis* males have a bare aedeagus making at least this sex distinct. Atchley (1967) examined populations of the *C. variipennis* complex in eastern New Mexico and reports finding no morphological evidence for

C. australis. This author synonymized *C. australis* with *C. sonorensis*; however, we have doubts as to what species Atchley (1967) actually examined (see below). Downes (1978) raised *C. occidentalis* to species status and tentatively list *C. australis* as a subspecies, though he did not discuss whether he agreed with the conclusions of Atchley (1967). Holbrook *et al*. (2000) examined the allotype male of *C. australis* and determined it to be *C. variipennis* due to the lack of spicules on the aedeagus; however, the determination label on this specimen says *C. sonorensis*. Additionally, this study also states that electrophoretic analysis of *C. australis* in Virginia were determined to be *C. variipennis*. It is unclear as to why *C. australis* was synonymized with *C. sonorensis* rather than *C. variipennis*.

Atchley (1967) provided measurements of the palpal ratio and number of sensilla celoconica as compared to the wing length of individuals collected from light traps in eastern New Mexico. He assumed that this series only represented *C. australis* and *C. sonorensis*, although *C. occidentalis* is also known from this area. The inclusion of this third species or even hybrid specimens may have biased the conclusions made in this study. Additionally, Atchley (1967) reared pupae from a salt lake in Loving, New Mexico and determined these to be *C. sonorensis*. In reexamining this material, we do not agree with this assessment as males from this series have spicules only at the distal end of the aedeagus indicating that they are potentially hybrids of *C. sonorensis* and *C. occidentalis*. However, this population does not fit the typical *C. australis* either, as females have only 1-2 extra sensilla celoconica and an extremely wide third palpal segment. As this location is outside of the range of *C. australis*, it is possible that these specimens represent hybrids of *C. occidentalis* and *C. sonorensis*.

[19, 20, 24, 25]

Culicoides combinothecus **Yu, Song, & Liu**

Culicoides combinothecus Yu, Song, & Liu, 1986:211. Type locality: Barkam County, Sichuan, China, 31°55'N, 102°15'E. Holotype ♀ slide (IMBC), 25 June, 1978.
Culicoides (*Monoculicoides*) *combinothecus*: Yu 2005:1269.

DIAGNOSIS: Male and female: only species of *C.* (*Monoculicoides*) with fine pubescence between the ommatidium.

DESCRIPTION: *Male*, **Head**: eyes separated by 2 ommatidia, with fine pubescence between each ommatidium. **Thorax:** scutum unknown; wings wings without pattern. **Abdomen:** epandrium strongly tapering posteriorly, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex, short; fused parameres with thick medially, short, stout base, apices widely separated; aedeagus triangular; gonostylus gradually tapering to midpoint. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2 ommatidia on the dorsal portion of the head, with fine pubescence between each ommatidium, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** spermatheca amorphous, slightly folded onto itself, with spermathecal ring present.

DISTRIBUTION: *Culicoides combinothecus* is known only from northern Sichuan, China.

Bionomics

Adult Habitat and Seasonality

The type locality of *C. combinothecus* lies within the Min Mountains and is around 2600 m elevation. All records of *C. combinothecus* are from June and July [26].

Immature Habitat

Unknown.

Feeding

With 13 fine teeth on the mandibles, *C. combinothecus* likely feeds on vertebrates [26].

Mating behavior

Unknown.

Development

Unknown.

Vector status

Unknown.

Molecular data

No barcode is available yet for this species.

Taxonomic Discussion

In the original species description of *C. combinothecus*, Yu *et al*. (1986) reports that this species is closely related to *C. parroti*, but can be distinguished by the presence of fine pubescence between the ommatidia and the shape of the spermatheca. Yu *et al*. (2005) recognized *C. combinothecus* as a member of *C*. (*Monoculicoides*); however, his key to the species of China contradicts Yu *et al*. (1986) by reporting *C. parroti* as also having fine pubescence between the ommatidia. The differences observed between these two works likely stems from the comparative material used. Yu *et al*. (1986) compared *C. combinothecus* to the descriptions of European *C. parroti*, whereas Yu (2005) compared this species to specimens of what they considered as *C. parroti* collected from China. Of the specimens we have examined of *C. parroti* throughout Europe, none have had the ocular pubescence described in Yu (2005). It may be that the absence or presence of these ocular setae is an expression of geographical variation within *C. parroti*, but this seems unlikely considering this type of variation is not known from any other broadly distributed species of *C*. (*Monoculicoides*) or other Ceratopogonidae. A more likely scenario is that the reports of *C. parroti* in central China are misidentifications of *C.*

combinothecus. *Culicoides parroti* females have an easily recognizable spermatheca, though when viewed dorsally (as in Gonzalez and Goldarazena (2011)), this structure looks very similar to what was described for *C. combinothecus*. Only one specimen of *C. combinothecus* has ever been reported and we believe this is due to the fact that the spermatheca of this species actually looks like that of *C. parroti*. The presence of the fine pubescence between the ommatidia seems to be the distinguishing characteristic of *C*. *combinothecus*; a feature unique within *C*. (*Monoculicoides*), and indicates that the *C. parroti* described in Yu (2005) are in fact *C*. *combinothecus*. Further examination of the Chinese material will likely clarify this interpretation.

Culicoides cornutus **de Meillon**

Culicoides cornutus de Meillon, 1937:332. Type locality: Blackburn, Zululand [South Africa]. Holotype ♂ slide (NMSA, lost?), 07 August 1936, reared from pupa; no Allotype designated; Paratypes ♂♀ slides (NMSA), same collection data; Paratypes ♂♀ slides (NMSA) Empangeni, Zululand [South Africa], 17 July 1936.
Culicoides (*Monoculicoides*) *cornutus*: Glick 1990:112.

DIAGNOSIS: Male: only Afrotropical species of *C*. (*Monoculicoides*) with gonostylus tapering gradually for basal half, with apical portion slender; female: only species of *C*. (*Monoculicoides*) with dense microsetae on the second and third palpal segments.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum yellowish brown with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 17-19 pleural setae; epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex; fused parameres with long, slender base, apices narrowly separated; aedeagus triangular, with numerous spicules; gonostylus tapering gradually for basal half, with apical portion slender.

Female, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 3-4 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with dense microsetae on second and third segments, third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 8-9; spermatheca ovoid, long, with spermathecal ring absent.

DISTRIBUTION: *Culicoides cornutus* is known from South Africa, Lethso, Namibia, Zimbabwe, Tanzania, and Kenya [27-33].

Bionomics

Adult Habitat and Seasonality

Culicoides cornutus occurs in the deserts shrublands, montane grasslands, and subtropical savannas of southern Africa . This species is considered rare in some areas [27, 34-36]; however, adults can be collected in high abundance on farms near animals pens, especially dairies [32, 37, 38]. This species has also been shown to have a relatively low dispersal ability [38] and so it stands to reason that they will only be collected in light trap directly adjacent to a larval breeding site. In South Africa, this species was collected in high abundance from light traps near a rhinoceros/impala enclosure [39]. In its southern distribution (Namibia, South Africa, Zimbabwe), this species is relatively absent from traps during the winter months [27, 28, 40]; however, in Kenya, *C. cornutus* is collected year-round [32, 38]. This species is most commonly collected between 1,200 and 1,800 m in elevation, with one collecting event at 700 m in South Africa and one collection at 2,300 m in Ethiopia [32, 36, 41]. An increase in species abundance is associated with an increase in rainfall in South and Africa and Kenya [37, 38]

Immature Habitat

Culicoides cornutus was described from specimens collected from a muddy rainwater pool, but no further details are given [42]. Many studies have found that the preferred larval habitat of this species to be mud mixed with a high degree of organic material, namely animal feces [37, 38, 43]. Interestingly, pupae of *C. cornutus* were rarely found in the Harare area of Zimbabwe even when surveying habitats high in organic material [29]. The *C. cornutus* pupae from this study were collected from muddy water near dams and we consider this to be an incidental collection rather than a true breeding site for this species. As *Culicoides* pupae float, they can easily be carried downstream, and if a dam is present, they will accumulate. Dams can be a great resource for surveying *Culicoides* species richness but offers little information in regards to larval habitat. Lubega & Khamala (1971) list the larval habitat of this species as both "aquatic" and "mud mixed with animal feces;" however, they define the aquatic system in a very broad context that ultimately encompasses multiple habitat types (water puddle, slow-flowing streams, and artificial effluent drainage trenches). This ambiguity seems to have artificially broadened the larval habitat to include all of these "aquatic" systems [34, 44].

Feeding

deMeillon 1937 noted that females of *C. cornutus* were biting at midday, but does not list a host. Precipitin test of blood meals recovered from engorged females collected in Kenya shows that this species feeds on sheep, goat, and cattle in farm settings, with one instance of an avian blood meal [38, 45]. Further blood meal analysis of specimens in a more natural setting could help to expand the host breadth of this species. Under laboratory conditions, wild-collected *C. cornutus* females readily fed on rabbit, guinea-pig, and human hosts [38].

Mating behavior

Males were observed forming mating swarms near breeding pools around midday, in direct sunlight [42].

Development

Walker and Davies 1971 collected a small number of individuals from the field and maintained these in the lab. Adults were fed on a sucrose solution and females were allowed to take a blood meal from a rabbit restrained to the top of the cage. Though numerous eggs were laid, larval mortality was extremely high. The author postulate that the lack of microbes in the larval substrate could have been the cause, though alternatively, obtaining a blood meal from an inadequate host could also be responsible [46, 47]. As is the case for most *C*. (*Monoculicoides*) spp., the preferred hosts of *C. cornutus* appears to be large ungulates and so the rabbit meal could have been insufficient for proper development. Of the surviving individuals, pupae were produced after 22 days. These authors also showed that *C. cornutus* was capable of taking at least two blood meals with an ovarian cycle of 8 days between feedings, indicating at the very least the vector potential of this species. Additionally, in Kenya, engorged parous females were collected and the number of parous individuals trapped was sufficiently high to allow for an estimated extrinsic incubation period of at least 15 days [45, 48].

Vector status

The main reasoning behind the investigation of the species as a vector lies in its close association to susceptible animals and its peak emergence times matching the presence of several diseases. Intensive serological testing has been carried out on the species in Kenya, yet the isolation of viral pathogens for bluetongue, Nairobi sheep disease, or African horse sickness have not been found in this species [38, 49]. Ephemeral fever virus has been isolated from pools for which *C. cornutus* constituted only 1% of the contents [49, 50]. In Delareyville, South Africa, epizootic

hemorrhagic disease virus was isolated from a pool of approximately 20 individual *C. cornutus* collected during an outbreak [51]. It is unclear if these pools contained whole bodies or just heads/salivary glands as this distinction can differentiate between the dissemination of the virus and the incidental ingestion of an infected blood meal. African horse sickness virus was not found in *C. cornutus* from South Africa, though only a few individuals were tested [41, 52]. While this species is often listed as a vector, there is very little evidence to support this and it is unlikely that it plays a pivotal role in disease transmission.

Molecular data

Barcodes are available for *C. cornutus* on Genbank (KY933278 - KY933282).

Taxonomic Discussion

The male, female, and pupa of *C. cornutus* were first described in deMeillon (1937) and this species was placed in *C.* (*Monoculicoides*) by Glick (1990). According to Segarman (1996) the holotype male should be at the NMSA; however, we are unable to confirm its location. As *C. cornutus* is the only species of this subgenus in southern Africa, it is readily distinguishable and several keys are available that highlight these differences [31, 33, 44, 53]. Partial descriptions of the adults of *C. cornutus* are present in all of these works; however, there are discrepancies in the descriptions of the male genitalia stemming from which specimens were examined. Khamala & Kettle (1971) examined one specimen from Kenya and reports that the caudal margin of tergite 9 is strait and the apicolateral processes are very long. Glick 1990 examined seven specimens from four countries and reports that the caudal margin of tergite 9 is concave and the apicolateral processes are broad. As we have examined all of the above specimens, we believe that these morphological differences constitute multiple species. The male specimen from Ethiopia is

describe as a new species later in the text. Interestingly, Glick examined both the specimens from Kenya and Ethiopia, yet did not to mention any of this variation.

[54]

Culicoides digitalis **Remm**

Culicoides (*Monoculicoides*) *digitalis* Remm, 1973:178. Type locality: Tsagaannuur district, Bayan-Ölgii Province, Mongolia. Holotype ♀ pinned (HNHM), "in the valley of the Chavcalyn gol [? river], 25 km east of Somon Cagannuur [Tsagaannuur district?], 1850 m, 3 June 1968 (No. 1056)"; Paratypes ♂ 1 pinned, ♀ 2 pinned (HNHM), same locality data, (No. 1056, 1057), ♀ 2 (HNHM), "Chovsgol aimak [Khövsgöl Province]: 8 km north of Somon Alag-erdene [Alag-Erdene district], on the Egijn gol [Egiin river], 1600 m, 17 July 1968, (No. 1119), ♀ 2 (HNHM), Chovsgol aimak [Khövsgöl Province]: "4 km northwest of the city of Mörön, 1500 m, 19 July 1968, (No. 1126)."

Culicoides xinghaiensis Yu, 1982:202. Type locality: Qinghai Lake, Xinghai County, Qinghai, China. No Holotype designated. **NEW SYNONYM**

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with a brown scutum and spicules on the aedeagus; female: only species of *C*. (*Monoculicoides*) with patterned wings and with a finger-like extension on the spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 3-4 ommatidia. **Thorax:** scutum brown with 3 indistinct light brown stripes; wings with faint pattern of pigmentation. **Abdomen:** abdominal segment 1 with 12 pleural setae; epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex; fused parameres with thick medially, short, stout base, apices narrowly separated; aedeagus triangular, long, with spicules only at apex; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 4 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow with small pit; mandibular teeth well

developed. **Thorax:** no difference**. Abdomen:** spermatheca spherical, with finger-like extension, with spermathecal ring present.

DISTRIBUTION: *Culicoides digitalis* is known only from the Mongolian provinces of Bayan-Ölgii, Khövsgöl, Övörkhangai, and Dornogovi, as well as, Qinghai Lake, China [26, 55].

Bionomics

Adult Habitat and Seasonality

Culicoides digitalis occurs in the desert and xeric shrublands of Mongolia and China [16]. All adult *C. digitalis* have been collected in June, July, and August and were collected using sweep nets along rivers and lakes [55].

Immature Habitat

As the adults can be collected along the banks of fresh-water rivers, we would assume that this would be the larval habitat [26, 55].

Feeding

Based on the finely serrate mandibles and retrorse teeth on the laciniae, *C. digitalis* females likely feeds on vertebrates [18].

Mating behavior

Unknown.

Development

Unknown.

Vector status

Unknown.

Molecular data

No barcode is available yet for this species.

Taxonomic Discussion

Other than catalogs, the description of *C. digitalis* in Remm (1973) is the only mention of this species in the literature. Remm (1973) also recognized this species as belonging to *C.* (*Monoculicoides*). We were unable to morphologically separate *C. xinghaiensis* from *C. digitalis*, and as such, have designated it as a synonym. In the original description of *C. xinghaiensis*, it was not compared to *C. digitalis* [56], nor has *C. digitalis* ever been reported from China [26, 57]. This is likely an oversite and the reason *C. xinghaiensis* was described as a new species. There are also only eight specimens of *C. xinghaiensis*, all collected from one area in Qinghai, China, and this province is not too far from the distribution of *C. digitalis*. Interestingly, *C. digitalis* was not included in any of the larger works reviewing the *C.* (*Monoculicoides*) of Russia [26, 58] and this could indicate that this species is confined to Mongolia and Inner Mongolia, China.

Culicoides expallens **Remm**

Culicoides (*Monoculicoides*) *expallens* Remm, 1973:176 Type locality: Bulgan aimak [Bulgan Province], Mongolia. Holotype ♀ pinned (HNHM), "5 km W of Somon Daschintschilen, 1140 m, 2 July 1964 (No. 252)"; Paratypes ♂ 2 slides pinned and slides, ♀ 68 pinned and slides (HNHM), from various locations in Mongolia (does not mention how many were from each location).

Culicoides erkaensis Yu and Yang 1988:135. Type locality: Manzhouli, Inner Mongolia, China. Holotype ♂ slide (IMBC), 13 August 1987. **NEW SYNONYM**

DIAGNOSIS: Male: only species of *C.* (*Monoculicoides*) with long apices of the parameres; female: only species of *C.* (*Monoculicoides*) with a lightly sclerotized spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 3-4 ommatidia. **Thorax:** scutum uniformly brown; wings with faint pattern of pigmentation. **Abdomen:** epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with narrow at

base, tapering to apex; fused parameres with thick medially, short, stout base, apices narrowly separated, long; aedeagus cylindrical, without spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow without defined pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 8-10; spermatheca ovoid, long, lightly sclerotized, with spermathecal ring absent.

DISTRIBUTION: *Culicoides expallens* is known from the Altai and Buryatia Republics of Russia, throughout the Mongolian steppe, south to Inner Mongolia, China [55, 58-61].

Bionomics

Adult Habitat and Seasonality

Gornostaeva (1986) considered this species to be confined to the steppe and forest-steppe zones of Mongolia and Russia, and all of the adult specimens have been collected in June, July, and August. All of the locations where this species has been collected are between 600-1600 m of elevation, with most being over 1000 m.

Immature Habitat

Remm (1973) collected individuals from the banks of rivers as well as at the margins of lakes. It is not apparent if these specimens were adults or immatures, though we would assume that these constitute the larval and pupal habitat. These include the Tuin and Ingoda River as well as Bayan lake, Buir lake, Khar-us lake, and Dus-Hol lake, the last of which is a salt lake.

Feeding

Unknown.

Mating behavior

Unknown.

Development

Gornostaeva (1985) studied the development and fecundity of 131 *C. expallens* females in the southern Transbaikal region of Russia. On average, each female collected had 169 ± 5 eggs and the number of eggs produced was directly proportional to the size of the individuals. Gornostaeva (1985) also found that females collected in June were larger than those collected in August and theorized that this constituted two separate generations. Perhaps the June females represent individuals that were overwintering as larvae, thus the larger size, and the August females are the offspring of that generation.

Vector status

Unknown.

Molecular data

None

Taxonomic Discussion

Remm (1973) recognized *C. expallens* as a member of *C*. (*Monoculicoides*) due to the presence of only a single spermatheca in the females and fused parameres in the males. The light sclerotization of the spermatheca in this species is unique within the subgenus; however, the male genitalia look very similar to that of *C. riethi* and *C. puncticollis*. Remm (1973) notes that *C. expallens* males can be separated from the species mention above by the long terminal extensions of both the parameres and aedeagus. We agree that this is a distinguishing feature of this species within *C*. (*Monoculicoides*). With this in mind, we believe that *C. erkaensis*, described in Yu (1988) is a synonym of *C. expallens*. From the drawings of the original description of *C. erkaensis*, the males have these same extended terminal ends of the parameres

and aedeagus. Yu (1988) compared the single specimen of *C. erkaensis* only to that of *C. homotomus*, and we assume that they were unaware of *C. expallens*. Additionally, the type locality of *C. erkaensis* (Manzhouli, China) borders both Mongolia and the Buryatia Republic of Russia, areas where *C. expallens* is known to be one of the most numerous species [55, 59]. In fact, Manzhouli, China is only about 150 km from where Gornostaeva (1985) conducted a study on *C. expallens* in which she specifically mentions its abundance in that region.

Culicoides grandensis **Grogan and Phillips**

Culicoides (*Monoculicoides*) *grandensis* Grogan and Phillips, 2008:197. Type locality: Grand Co., Utah, USA. Holotype ♂ slide and Allotype ♀ slide (USNM), on slides labeled ‘‘Utah, Grand Co., near Cisco, margin alkaline stream, 30-V-1958, R. H. Jones, Jones No. 3661 (holotype), No. 3660 (allotype)’’; their respective pupal exuviae with same data with probable emergence date of ‘‘20-VI-1958, Jones No. 3661 (holotype), No. 3660 (allotype); Paratype ♀ slide-mount (CNCI), on slide labeled ‘‘Utah: Grand Co., 4 km SW Moab, CDC LT, 18-IX-01, coll: R. A. Phillips’’.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with a brown scutum, with apices of parameres on medial neck, and with a cylindrical aedeagus; female: only species of *C*. (*Monoculicoides*) without teeth.

DESCRIPTION: *Male*, **Head**: eyes separated by 2 ommatidia. **Thorax:** scutum brown with 2 light brown stripes; wings with faint pattern of pigmentation. **Abdomen:** epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex; fused parameres with long, slender base, apices nearly touching on medial neck; aedeagus cylindrical, without spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow without defined pit; mandibular teeth absent. **Thorax:** no

difference**. Abdomen:** abdominal segment 1 with 3; spermatheca spherical, with spermathecal ring absent.

DISTRIBUTION: *Culicoides grandensis* is known only from the northwest portion of Utah, USA.

Bionomics

Adult Habitat and Seasonality

Grogan and Phillips (2008) proposed that the range of *C. grandensis* might extend to other parts of the Colorado Plateau, but this remains unknown. This species was reared from the larval habitat in late May and an individual was collected in a CDC light trap September 18 [62].

Immature Habitat

Culicoides grandensis (as a n. sp.) was reared from the shoreline of a shallow alkaline stream with white salt deposits above the margins [63]. One side of the habitat was heavily vegetated with tall grasses and the opposite side was bare and in constant direct sunlight (Fig. 3.1). Other *Culicoides* species were also reared from this habitat, including *C. jamesi*, *C. haematopotus*, *C. occidentalis* or *C. sonorensis* (as *C. variipennis*), *C. crepuscularis*, and *C. stonei*.

Figure. 3.1. The larval habitat of *C. grandensis*, near Cisco, Utah.

Feeding

Female adults of this species are presumed to either not feed or feed solely on sugar sources, due to the lack of mandibular teeth.

Mating behavior

Unknown.

Development

Unknown.

Vector status

Seemingly, *C. grandensis* would not have a role in pathogen transmission since it does not appear to take blood meals.

Molecular data

Unknown.

Taxonomic Discussion

As only three specimens of this species exist, not much is known about the biology of this species. Morphologically, *C. grandensis* has several adult and pupal characters unique within the subgenus. The complete loss of mandibular teeth in the female, non-bifurcated aedeagus in the male, and bare dorsal apotome in the pupa are only found in this species. In the original species description, adult morphology of *C. grandensis* was compared to that of *C. riethi* (as *C. gigas*), *C. stigma*, and the *C. variipennis* complex [62]. The pupa of *C. grandensis* is described in Shults and Borkent (2018). In July 2020, we attempted to collect specimens from the same stream in Utah where Jones collected the first specimens. Unfortunately, were unable to collect any *C. grandensis*, though we did find numerous *C. occidentalis* and *C. sonorensis* pupae.

[10, 64]

***Culicoides heiheensis* Li, Zhang and Liu**

Culicoides heiheensis Li, Zhang and Liu, 2011:363. Type locality: Heihe, Heilongjiang, China. Holotype ♂ slide (Entomology Collection Gallery of Heilongjiang Entry-Exit Inspection and Quarantine Bureau, Harbin 150001 ,China).

Culicoides aihuiensis Wu, Jiao and Lui, 2019:76. Type locality: Heihe, Heilongjiang, China. Holotype ♂ slide (Center for Disease Control and Prevention of Shenyang Comnmand), "from the Aihui District if Heihe City, 15 July 1982." **NEW SYNONYM**

Culicoides (*Monoculicoides*) *heiheensis*: Borkent 2020:117.

DIAGNOSIS: Male: only species of *C.* (*Monoculicoides*) with the width of parameres greater than the length and with caudal margin of the epandrium slightly concave; female: unknown.

DESCRIPTION: *Male*, **Head**: eyes separated by 2 ommatidia. **Thorax:** scutum unknown; wings with faint pattern of pigmentation. **Abdomen:** abdominal segment 1 with unknown number of pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process with narrow at base, tapering to apex; fused parameres with squatty, apices widely separated; aedeagus triangular, without spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, unknown.

DISTRIBUTION: *Culicoides heiheensis* is known only from Heihe, Heilongjiang, China [65, 66].

Bionomics

Adult Habitat and Seasonality

The ecoregion of China where *C. heiheensis* occurs is a Manchurian mixed forest [16].

Immature Habitat

Unknown.

Feeding

With 15 fine teeth on the mandible [65, 66], *C. heiheensis* likely feeds on vertebrates [18].

Mating behavior

Unknown.

Development

Unknown.

Vector status

Unknown.

Molecular data

None

Taxonomic Discussion

In the original description of *C. heiheensis*, Li *et al*. (2011) noted the similarities of the male genitalia of this species to that of *C. longlinensis*, but report that *C. heiheensis* has a significantly more pigmented patterning on the wing. It appears however, that what Li *et al*. (2011) referred to as the aedeagus is actually the parameres, and it is not clear what structure they drew when describing the parameres. Due to this ambiguity, Wu *et al*. (2019) were unable to recognize *C. heiheensis* and described their new species *C. aihuiensis*, also from Heihe, China, with the main differences listed between these species being the male genitalia. Considering the exact same type localities and morphological similarities, we consider *C. aihuiensis* to be a junior synonym of *C. heiheensis*. It should be noted that description of *C. aihuiensis* in Wu *et al*. (2019) is a more accurate and complete description of the male of this species. *Culicoides heiheensis* clearly belongs to *C*. (*Monoculicoides*) due to the dark spot just posterior to the arculus and M_3+CuA vein on the wing, medially fused parameres, and bifurcate aedeagus. Within this subgenus, *C. heiheensis* and *C. longlinensis* are the only two species where the parameres are twice as wide as they are long.

Culicoides helveticus **Callot, Kremer & Deduit**

Culicoides helveticus Callot, Kremer & Deduit, 1962:164. Type locality: Canton de Vaud, Switzerland. Holotype ♀ pinned (IPSF), "Combe des Amburnex, [September 1961], au bord d'un gouffre [at the edge of a chasm]."

Culicoides (*Monoculicoides*) *helveticus*: Glukhova 1979:233.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with wings lacking pattern of pigmentation, with distinct posterior projection medial to base of apicolateral process, and with gonostylus tapering gradually for basal half, with apical portion slender; female: only species of *C*. (*Monoculicoides*) with a brown scutum and a finger-like extension on the spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2 ommatidia. **Thorax:** scutum dark brown with lighter hour-glass pattern on posteromedial area; wings without pattern of pigmentation. **Abdomen:** abdominal segment 1 with 8 pleural setae; epandrium with nearly parallel lateral margins, with distinct posterior projection medial to base of apicolateral process, half as long as apicolateral process; apicolateral process with narrow at base, tapering to apex; fused parameres with long, slender base, apices widely separated; aedeagus triangular, without spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 3-8; palpus with third segment narrow without defined pit; mandibular teeth well developed. **Thorax:** no difference**.** **Abdomen:** spermatheca spherical, with finger-like extension, with spermathecal ring present.

DISTRIBUTION: *Culicoides helveticus* is known from southern Sweden and Estonia to Ukraine, east to the far eastern districts of Russia and south to northern Mongolia and China, with scattered populations known from the Alps (France, Switzerland, and Germany), the Carpathian mountains (Romania), and the lower Caucus mountains (Georgia). [26, 55, 58, 67-83].

Bionomics

Adult Habitat and Seasonality

In its western distribution*, C. helveticus* occurs primarily in the alpine and sub-alpine forests of mountainous regions [58, 70]. This species can also be collected throughout the boreal forests of the taiga region of Russia [58, 84], though it is rare in the forest tundra above 68°N [77]. Because of this distribution, reports of *C. helveticus* from Spain, Morocco, and Sardinia are listed

as doubtful [85, 86]. Adults can be collected during the summer months and are reported as being common, yet rarley reaching high abundance [58, 69, 76].

Immature Habitat

Culicoides helveticus larvae can be found in silty deposits of forest reservoirs and muddy stream overflows [75, 78, 87]. In a survey of the *Culicoides* breeding habitats of southern Siberia and Far East Russia, Glushchenko and Mirzaeva (2008) considered *C. helveticus* to be a eurytopic species breeding in a variety of habitats including man-made reservoirs, spring bogs, rivers flowing between mountain basins, brooks, and temporary floodplain reservoirs. Immature *C. helveticus* have been collected with *C. stigma* and *C. riethi* [88].

Feeding

While studying *C. helveticus* in the Lesser Khingan mountains of Russia, Katsko (1975) reported the females of this species attacking humans; however, this was likely due to the uncommonly high densities. In southern Siberia where this species is rarer, Mirzaeva (1969) never observed this species feeding on humans.

Mating behavior

Unknown.

Development

In southern Siberia, the larvae, and occasionally pupae, were found with significant infections of mermithid nematodes [78, 89].

Vector status

Unknown.

Molecular data

None.

Taxonomic Discussion

Callot *et al*. (1962) described the adult female of *C. helveticus* in great detail, yet only included a small paragraph on the male. Kremer (1965) provided redescriptions of the adults of this species with a much more detailed description of the males, including figures. Additional descriptions of the adults can be found in Glukhova (1989) and Yu (2005), both of which are in general agreement with our assessment of this species. While both Callot *et al*. (1962) and Kremer (1965) placed this species in the *stigma-nubeculosus* group, it is unclear if these authors were referring to the *nubeculosus* group within *C*. (*Monoculicoides*). In describing the larvae of *C. helveticus*, Glukhova (1979) was the first to clearly assign this species to the subgenus *C*. (*Monoculicoides*). *Culicoides helveticus* larvae can be distinguished from all other Palearctic species of this subgenus, except for *C. stigma*, because of its yellow head and widely spaced sensillae on labrum. The pupa of *C. helveticus* was described by Glukhova (1989).

***Culicoides homotomus* Keiffer**

Culicoides homotomus Kieffer, 1922:158. Type locality: Formosa [= Taiwan]. Holotype ♀ (possibly at the BGBM, not at HNHM).

Culicoides osakensis Iwata, 1935:7. Type locality: Osaka, Japan. Syntype ♂ and ♀ (location unknown); Tokunaga, 1937:280, as a synonym of *C. nubeculosus*; Vargas, 1949:10, as a new synonym.

Culicoides nubeculosus: Tokunaga, 1937:280: (misidentification). Not *C. nubeculosus* (Meigen 1830):263, from Kagi, Formosa [Taiwan], Vargas, 1949:10.

Culicoides denmeadi Causey, 1938:401. Type locality: Chiang Rai, Siam [Thailand]. Holotype ♀ slide (USNM); Wirth and Jones, 1957:3, as a new synonym.

Culicoides buhetoensis Takahashi, 1941:81. Type locality: Buheto, Manchuria [Heilongjiang province, People's Republic of China]. Holotype ♀ (EIHIU). **NEW SYNONYM**.

Culicoides obtusus Chatterjee, Brahma & Hazra, 2020:24. Type locality: Narayanpur, West Bengal, India. Holotype ♂ slide (NZCI); Paratypes 1♂, 3♀ slides (NZCI), same collection data; 2♂, 1♀ slides (Burdwan University Entomology Division), with pupal exuviaum, labelled as "Paratype *Culicoides* (*Monoculicoides*) obtusus Chatterjee, Brahma & Hazra, India, West Bengal, Dakshin Dinajpur, Kushmundi [25°52′61″N, 88°35′76″E], 24.IV.2017, Coll. P. Saha". **NEW SYNONYM**.

DIAGNOSIS: Male and female: only species of *C*. (*Monoculicoides*) distributed in the Oriental region though range overlaps with some Palearctic species. In addition, male: only species of *C*. (*Monoculicoides*) with light brown scutum with black spots; female: only species of *C*. (*Monoculicoides*) with the second palpal segment slightly enlarged.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax**: scutum light brown with black spots; wings with well-defined pattern of pigmentation. **Abdomen**: abdominal segment 1 with 16-20 pleural setae; epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex; fused parameres with thick medially, short, stout base, apices widely separated; aedeagus triangular, without spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with second segment slightly enlarged, third segment narrow with small pit; mandibular teeth well developed. **Thorax**: no difference. **Abdomen**: abdominal segment 1 with 15-17; spermatheca ovoid, with spermathecal ring absent.

DISTRIBUTION: *Culicoides homotomus* is known from India, China, Taiwan, South Korea and from the Japanese islands of Honshu and Kyushu, south to Cambodia, Thailand, and Malaysia [90-111].

Bionomics

Adult Habitat and Seasonality

Culicoides homotomus occurs in the tropical and subtropical moist broadleaf forests of southeast Asia as well as the temperate broadleaf and mixed forest from China to Japan [16]. The adults of this species are often associated with livestock farms and can be collected regularly at light traps

near large mammals. In India, this species was collected in December to May [109, 110]. This species was collected at low abundance nearly year round in Fujian, China [99] and from March to November in Jiangxi, China [103]. In Taiwan, this species was collected in high abundance in the summer months; however, there were also peak emergences in February and March [112]. In South Korea, *C. homotomus* was collected predominantly from May-July with population densities varying considerably by year [90, 92].

Immature Habitat

Iwata (1935) reported the larvae from a small pool of fresh-water. Takeda & Mukai (1954) observed the eggs (as *C. nubeculosus*) laid at the margin of a sewage ditch. Chatterjee *et al*. (2020) collected a pupa of *C. homotomus* from a pond that feeds into a drainage system on a cow farm.

Feeding

Female adults been observed directly feeding on water buffalo in Taiwan [112], and blood meal analysis from specimens collected in South Korea showed feeding on both cattle and chickens [91]. In the Anhui province of China, *C. homotomus* was the dominant species collected from farms raising buffalo, yellow cattle, dairy cattle, donkeys, goats, sheep, and pigs [108]. It was also collected from cow and pig sheds in Jiangxi, China, but were also readily collected from other habitats including parks and residential areas [103]. Interestingly, this species was only collected at low abundance from cattle sheds (what should be a preferred habitat) in both South Korea and India [92, 110]. There are also reports of this species biting man in Japan, especially in coastal areas [113-115].

Mating behavior

Unknown.

Development

Engorged females were collected from the field and allowed to lay eggs on an artificial substrate under laboratory conditions. The eggs hatched but died during the 1st instar stage [116]. Add Jeu 1974 and 1977.

Vector status

Like many other *C.* (*Monoculicoides*) species, the larval habitat and feeding preference puts *C. homotomus* in close proximity to livestock. Zhang (1995) found that the peak emergence time and latent period of this species coincided with the epidemic period of bluetongue in southern China. Additionally, an unidentified RNA virus was isolated from *C. homotomus* collected in China [117]. When injected intravenously this virus induced an antibody response in cattle with no clinical signs; however, infected sheep developed severe fever. This virus is likely to be a strain of BTV. Yanase (2005) screened Japanese *Culicoides* for the viral isolates of several diseases and none were present in the single specimen of *C. homotomus* tested. The presence of several *Orthobunyavirus* antibodies were detected in cows in Jeju island, Korea, and though *C. homotomus* was abundant, no viral antibodies were found in this species. Takeda & Mukai (1954) reported an outbreak of a skin disease in a small coastal village in Japan and attributed it to the bites of this species (as *C. nubeculosus*). Poor sanitation was determined to be the cause of the increased biting midge population, and when the sewage ditches were drained, disease incidence subsided.

Molecular data

Partial COI sequences are available for specimens collected in China and Thailand [96, 103] and the overall genetic similarity between these two locations was high.

Taxonomic Discussion

After the original species description from Taiwan, Iwata (1935) described specimens from Osaka Japan as *C. osakensis*. Tokunaga (1937) misidentified material collected from Japan and synonymized this species with *C. nubeculosus* as he was unable to find any differences between the two thus he regarded *C. osakensis* as a synonym. Vargas (1949) later determined that both of these designations were referring to *C. homotomus* and recognized *C. osakensis* as a synonym of *C. homotomus*. Causey (1938) described a single female from Thailand as *Culicoides denmeadi*, but this was later synonymized with *C. homotomus* by Wirth and Jones (1957). Wirth & Jones (1957) also placed *C. homotomus* into the subgenus *C*. (*Monoculicoides*). Takahashi (1941) described a single female as *C. buhetoensis* from northeast China; however, we are unable to distinguish between its description and *C. homotomus* and formally recognize the synonymy here. Chatterjee *et al*. (2020) also described a new species, *C. obtusus,* an similarly, we are unable to differentiate the description of this species from *C. homotomus*. Thus, both of these designations have been listed as new synonyms here.

Re-descriptions of male and female adult *C. homotomus* can be found in Arnaud (1956), Wirth and Hubert (1961), McDonald and Lu (1972) (female only), Wirth and Hubert (1989), Wang (2002), and Yu (2005). Generally, all of these descriptions are in agreement with each other as well as with our morphological comparison. The most apparent differences are in Causey (1938) and McDonald and Lu (1972) which describe the scutum as dark brown with not mention of black or brown dots. This is a relatively striking feature and so it seems odd to omit, so perhaps these specimens were damaged. The egg, larvae, and pupa are described in Jeu and Rong (1981) [in Chinese] with morphological comparisons to *C. nubeculosus*.

A new species of *C*. (*Monoculicoides*), *C. obtusus*, was described from West Bengal, India in by Chatterjee *et al*. (2020). Their description appears very similar to that *C. homotomus* but the

authors do propose characters to separate the two species: presence of a distinct pale spot in R3 in both the male and females, shape of the aedeagus, position of the lateral arms of the paramere, size of the spermatheca, and number of pores of the pupal respiratory organ. The wings of *C. homotomus* and *C. obtusus* are shown side-by-side on their page 17 and indeed look slightly different; however, the wing of *C. obtusus* is identical to the species description of C. homotomus in Arnaud (1956). Additionally, this slight variation in pigmentation can arise simply from geographic, seasonal, or other environmental factors. Chatterjee *et al*. (2020) also include a figure comparing the male genitalia of these two species. The specimen of *C. homotomus* pictured there is clearly crushed under a coverslip on the slide; distorting many of the morphological characters. This specimen also seems to be the one used for comparative purposes as the characters listed for *C. homotomus* in the key are similar to this specimen. The *C. homotomus* we examined do not match the diagnostic characters used in their key. Size of the spermatheca as listed as a character used to separate these two species; however, our measurements of *C. homotomus* overlap with the size of the spermatheca reported for *C. obtusus*. The pupa of *C. obtusus* is also described and though the authors allude to multiple pupal characters differentiating it from *C. homotomus*, only the number of respiratory pores is listed. It is also not clear how the authors knew what the pupa of *C. homotomus* looked like as they did not cite the pupal description in Jeu (1981). Presumably, they collected and reared individuals from both species to compare but this is not listed in the methods. Both Jeu (1981) and Chatterjee *et al*. (2020) list three lateral respiratory pores so the only difference between these two descriptions is the number of terminal respiratory pores reported (13-14 and nine respectively). The number respiratory openings on the respiratory horn can vary within a species and sometimes even on the same specimen [10, 118, 119]. In comparing our material of *C.*

homotomus to the descriptions of *C. obtusus*, as well as the species key in Chatterjee *et al*. (2020), we are unable to distinguish these two species and therefore consider *C. obtusus* as a new synonym of *C. homotomus*.

[112, 120]

***Culicoides lochmocola* Yu, Ayiken & Chen**

Culicoides lochmocola Yu, Ayiken, & Chen, in Chen *et al.,* 2016:583. Type locality: Fukang, Xinjiang, China. Holotype ♂ slide (Beijing Medical Insect Herbarium), 01 September 2015.

Culicoides (*Monoculicoides*) *lochmocola*: This study. **NEW STATUS**

DIAGNOSIS: Male: only species of *C.* (*Monoculicoides*) with a patterned wing and with distinct posterior projections medial to base of apicolateral process; female: unknown.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum unknown; wings with faint pattern of pigmentation. **Abdomen:** abdominal segment 1 with unknown number of pleural setae; epandrium with nearly parallel lateral margins, with distinct posterior projection medial to base of apicolateral process, less than half as long as apicolateral process; apicolateral process with narrow at base, tapering to apex; fused parameres with stout base, apices widely separated; aedeagus triangular; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, unknown.

DISTRIBUTION: *Culicoides lochmocola* is known only from the northern Xinjiang, China [121].

Bionomics

Adult Habitat and Seasonality

The type locality for *C. lochmocola* is in a desert and xeric shrubland [16]. The one specimen of *C. lochmocola* was collected September 01, presumably from a light trap.

Taxonomic Discussion

Other than a brief description of the male, no further information is available for this species.

***Culicoides longicollis* Glukhova**

Culicoides longicollis Glukhova, 1971:507. Type locality: Truskavets, Lviv Oblast, Ukraine. Holotype ♀ slide (ZIN), "April 1966, pupae and larvae in mass in a brook polluted by wastes from the municipal dump"; Paratypes 9 ♂ slides, ♀ 14 slides, 5 P,L slides, same collection information.

Culicoides (*Monoculicoides*) *longicollis*: Glukhova, 1979:229.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with the apices of parameres on medial neck and with spicules on a cylindrical aedeagus; female: only species of *C*. (*Monoculicoides*) with a slightly curved spermatheca and a uniform spermathecal duct at its opening.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 20 pleural setae; epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with narrow at base, tapering to apex; fused parameres with thick medially, short, stout base, apices tips long, nearly touching; aedeagus cylindrical, with numerous spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 4-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 15-20; spermatheca ovoid, with slight curve near base, with spermathecal ring absent.

DISTRIBUTION: *Culicoides longicollis* is known from Poland, Slovakia, and the Ukraine, south to Armenia, east to Kazakhstan and Kyrgyzstan [58, 84, 122, 123].

Bionomics

Adult Habitat and Seasonality

All of the recorded collections of C. *longicollis* have been in April and May with one specimen collected in October from western Turkmenistan. The ecoregions where this species occurs are temperate grasslands, savannas and shrublands, and xeric shrublands [16]. Glukhova (1989) states that adults of this species are wide-spread throughout eastern Europe and Central Asia, but are not numerous in these places.

Immature Habitat

The holotype and paratypes of C. *longicollis* were collected from a brook polluted by waste [123]; however, Szadziewski (1983) provided evidence that this habitat may have been saline. In Poland, C. *longicollis* was one of the most common species found in strongly saline, inland areas [124]. The larval density in these ponds increases 5-fold from October to May as this likely represents the time in which this species overwinters.

Feeding

This species has been reported to attack cattle, muskoxen, and humans [58, 123].

Mating behavior

Unknown.

Development

Glukhova (1989) reports the species as anautogenous [58].

Vector status

Unknown.

Molecular data

None

Taxonomic Discussion

Glukhova (1971) includes descriptions of the male, female, and larvae of *C. longicollis*, and as it closely resembles *C. nubeculosus*, recognized this species as part of the *C. nubeculosus* group. Redescriptions of the male, female, and larval stages can be found in Glukhova (1979), which also formally moved this species to *C*. (*Monoculicoides*). Additional descriptions of the larvae are in Glukhova (1977) and Szadwiewski *et al*. (1997) [125, 126]. There are significant differences between the male and female genitalia of *C. longicollis* and *C. nubeculosus*; however, the larvae and pupae appear to be indistinguishable [123, 127].

***Culicoides longlinensis* Yu**

Culicoides longlinensis Yu, 1982:202. Type locality: Longlin County Guangxi, China. Holotype ♂ slide (IMBC), collected in 1957.
Culicoides paradoxus Yu and Liu, 1990:2. Type locality: Nanchang, Jiangxi, China. Holotype ♂ slide (IMBC).
Culicoides (*Monoculicoides*) *longlinensis*: Yu 2005:1273.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with width of the parameres greater than length and with caudal margin of the epandrium deeply concave; female: only species of *C*. (*Monoculicoides*) with third palpal segment narrow with small pit, faint wing patterning, and with an ovoid spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum unknown; wings with faint pattern of pigmentation. **Abdomen:** epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with narrow at base, tapering to apex; fused parameres with squatty, apices narrowly separated; aedeagus not drawn;

gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**.** **Abdomen:** spermatheca ovoid, with spermathecal ring absent.

DISTRIBUTION: *Culicoides longlinensis* is known only from the Guangxi and Jiangxi provinces of China [26].

Bionomics

Adult Habitat and Seasonality

The ecoregion of China where C. *longlinensis* is found is a subtropical broadleaf forest [16].

Feeding

With 14 fine teeth on the mandible [56], female adult *C. longlinensis* likely feed on vertebrates. Little else is known about this species.

Taxonomic Discussion

The specimens used to describe the adults of *C. longlinensis* were collected in 1957 from Guangxi, China. *Culicoides paradoxus* was described in Yu and Lui (1990) from specimens collected in Jiangxi, China. In recognizing *C. longlinensis* as a member of *C.* (*Monoculicoides*), Yu (2005) also recognized that *C. paradoxus* was a synonym of *C. longlinensis*. Yu (1982) notes that the females of this species look similar to that of *C. riethi* and *C. homotomus*, though *C. longlinensis* is reported to be much smaller and have less pigmentation on the wing [26, 56, 128]. The male genitalia of C. *longlinensis* are nearly unique within *C.* (*Monoculicoides*) with the parameres being twice as wide as they are long. This feature is only seen in one other species within this subgenus, *C. heiheensis*.

Culicoides mullensi n. sp. Shults and Borkent

Culicoides (*Monoculicoides*) *mullensi* Shults and Borkent. Type locality: San Diego, California, USA. Holotype ♀ slide-mount (USNM), labeled "collected from light trap, 32.5522, -117.0628, July 11, 2014"; Paratypes 3 and 5 ♀ slides, (CNCI, USNM), labelled "KOH-Balsam -R.H. Jones, Hueneme 20 VI-48, Ventura Co, California, salt marsh, W.W. Wirth. **NEW SPECIES**

DIAGNOSIS: Male and female: only species of *C*. (*Monoculicoides*) with prominent black spots on scutum and brown vittae, and sensilla coeloconica present on six or more flagellomeres. In addition, male: only species of *C*. (*Monoculicoides*) caudal margin of S9 straight, and with aedeagus bare; female: only species of *C*. (*Monoculicoides*) with third palpal segment wide.

DESCRIPTION: Male, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots and brown vittae; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 22-24 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process with narrow at base, tapering to apex; fused parameres with long, stout base, apices narrowly separated; aedeagus triangular, without spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 3-4 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 3-8; palpus with third segment wide with large pit; mandibular teeth well developed. **Thorax:** no differences**. Abdomen:** abdominal segment 1 with 22-25 pleural setae; spermatheca ovoid, long, C-shaped, spermathecal ring absent.

DISTRIBUTION: *Culicoides mullensi* is known from Lake County, California south along the coast to San Diego, California, USA.

Bionomics

Adult Habitat and Seasonality

The region of California where *C. mullensi* is found is a mediterranean forests, woodlands, and scrub . All of the known specimens of *C. mullensi* were collected from April to July. The slide mounted material of *C. mullensi* comes from specimens collected near the coast in southern California. A blast search of COI sequences from this species, obtained in chapter II, show that *C. mullensi* occurs at least as far north a Lake County California.

Immature Habitat

W.W. Wirth reared a series of *C. mullensi* from a salt marsh pool in Hueneme, California.

Feeding

Unknown

Mating behavior

Unknown

Development

Unknown

Vector status

Unknown

Molecular data

COI sequences for *C. mullensi* are available; however, only those listed in chapter II have been identified using SNP markers. Sequences in GenBank with >99.0% similarity to these samples are as follows: JF870510.1 from Camarillo, California (as *C. sonorensis*), KY707858.1 from Middletown, California (as *C. variipennis* complex sp.), KY707839.1 from Clearlake Oaks, California (as *C. occidentalis*), and KY707873.1 from Spring Valley, California (as *C. variipennis* complex sp.)

Taxonomic Discussion

Culicoides mullensi is morphologically and ecologically very similar to *C. occidentalis*. In the original subspecies designation of *C. occidentalis*, Wirth and Jones (1956) highlight several unique looking series of specimens from southern California, designated as paratypes here. They note that females from these sites have more flagellomeres with sensilla coeloconica and are much bigger and darker than most *C. occidentalis*. They also state that they "doubtfully refer to [these] as *occidentalis* on the basis of distribution." This alone may not have warranted full species status; however, in combination with the molecular results found in chapter II, we feel confident that these specimens represent a new species. We agree with the assessment of Wirth and Jones (1956) that the only morphological differences between *C. mullensi* and *C. occidentalis* are size and the number of flagellomeres with sensilla coeloconica. Because of this overall similarity, *C. mullensi* should be considered as part of the *C. variipennis* complex. Both SNP and mitochondrial data provide further evidence of the validity of this species. Genetically, *C. mullensi* is just as divergent from *C. occidentalis* as *C. occidentalis* is from *C. sonorensis* or *C. variipennis*. We consider *C. mullensi* to be a member of the *C. variipennis* complex.

This species is named after Bradley Mullens who has worked on the *C. variipennis* complex throughout his career and has provided the *Culicoides* community with a wealth of biological, ecological, and epidemiological data.

Culicoides nanpingensis Yu, Ayiken & Chen,

Culicoides nanpingensis Yu and Song, 1986:209. Type locality: Nanping County, Chongqing, China. Holotype ♀ slide (IMBC), collected by light trap, August 1978, (29°10'N, 106°54'E).

Culicoides (*Monoculicoides*) *nanpingensis*: Yu, 2005:1274.

DIAGNOSIS: *Male*: unknown. F*emale*: only species of *C.* (*Monoculicoides*) with wings lacking a pattern of pigmentation and with a spherical, unmodified spermatheca.

DESCRIPTION: *Male*, unknown. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 4-8; palpus with third segment narrow without defined pit; mandibular teeth well developed. **Thorax:** unknown. **Abdomen:** spermatheca spherical, with spermathecal ring absent.

DISTRIBUTION: *Culicoides nanpingensis* is known only from the Chongqing Province of China [26, 129].

Bionomics

Adult Habitat and Seasonality

Yu *et al*. (1986) mentions the specimens collected in that study were from the north-west region of the Sichuan province of China; however, the coordinates of the holotype are from the Chongqing Province. We assume that the actual habitat of this species is that of the later. The coordinates are in Nanping county which is part of the subtropical broadleaf forest ecoregion.

Feeding

With 14 fine teeth on the mandibles [26], *C. nanpingensis* likely feeds on vertebrates.

Little else is known about this species.

Taxonomic Discussion

Culicoides nanpingensis was described from a single female collected in Yu *et al*. (1986) and placed into *C*. (*Monoculicoides*) by Yu (2005). Yu *et al*. (1986) note this species similarity to *C. stigma*, but reports that it can be distinguished by the lack of the finger-like extension off of the spermatheca. We would agree with this comparison and have placed *C*. *nanpingensis* into the *C. stigma* group because of the lack of patterning on the wing, subequal antennal ratio, and the presence of a faint spermathecal ring.

Culicoides nubeculosus (Meigen)

Ceratopogon nubeculosus Meigen, 1830:263. Type locality: Europe, no holotype designated.
Culicoides puncticollis Goetghebuer, 1912: 205 (preoccupied by *Culicoides puncticollis* (Becker, 1903)). Destelbergen, Belgium.
Culicoides punctaticollis Goetghebuer, 1920: 56. New name for *puncticollis* Goetghebuer.
Culicoides (*Monoculicoides*) *nubeculosus*: Khalaf 1954:40.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with the apices of parameres on medial neck and with gonostylus gradually tapering distally; female: only species of *C*. (*Monoculicoides*) with a slightly curved spermatheca and a swollen spermathecal duct at its opening.

DESCRIPTION: *Male*, **Head**: eyes separated by 3-4 ommatidia. **Thorax:** scutum grey with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 19-22 pleural setae; epandrium with strongly tapering posteriorly, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex; fused parameres with short, stout base, apices nearly touching on medial neck; aedeagus cylindrical, without spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 4 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 4-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 16-21; spermatheca ovoid, with slight curve near base, with spermathecal ring absent.

DISTRIBUTION: *Culicoides nubeculosus* is broadly distributed throughout most of Europe and Russia, from the United Kingdom to Siberia, south to Spain and Turkey [58, 84, 130-139].

Bionomics

Adult Habitat and Seasonality

The adults of *C. nubeculosus* can be found in high densities on livestock farms [140] and are active from May to October [135]. This species occurs primarily in the temperate and boreal forests of Europe and Russia [16].

Immature Habitat

The larvae of *C. nubeculosus* breed in water contaminated with organic material such as animal manure [15, 130, 141, 142]. Kettle and Lawson (1952) also collected a few immature *C. nubeculosus* from a salt marsh in the United Kingdom.

Feeding

Culicoides nubeculosus females attack a variety of livestock animals such as cattle, sheep, and horses [130, 143], and primarily feed on the withers and hindquarters [144]. Evidence suggests tha many *Culicoides* species that feed on large mammals use similar cues for host seeking [145], though Isberg (2016) isolated volatiles within the hair and urine of cattle that elicited attraction from female *C. nubeculosus*.

Mating behavior

In nature, *C. nubeculosus* males have been observed swarming near the larval habitat using dark colored material as swarming markers, and on occasion, mating at the host [146, 147]. Downes (1955) gives a detailed description of the swarming behavior and swarm morphology of this species. Additionally, *C. nubeculosus* are able to mate within confined spaces which has led to the colonization of this species [148]. Female *C. nubeculosus* produce a sex pheromone which the males then use to find the females [149, 150]. Mating appears to occur after contact and in many cases, the females show at least some level of resistance to mating [151]. This may be so that she can assess the fitness of the males based on their mating persistence. Recently mated

females are highly resistant to further matings, however, as the female ages, her receptiveness to copulation increases [152].

Development

Laboratory colonies of *C. nubeculosus* have been established and the developmental times of each life stage have been measured [140, 148].

Vector status

Evidence from laboratory trials have shown the *C. nubeculosus* females are not likely to be involved in the transmission of arboviruses [153-155]. Replication of BTV, Akabane virus, Schmallenberg virus, and AHS has been reported inside this species after intrathorasic infection, though transmission levels are generally very low [156-160]. *Culicoides nubeculosus* females do however seem to be responsible for insect bite hypersensitivity and dermatitis in horses, as well as, the transmission of *Onchocerca* [161]. *Haemoproteus* species can also be transmitted by *C. nubeculosus* though as birds are not this species primary host [162, 163], its role in transmission in appears minimal.

Molecular data

Both mitochondrial and nuclear sequences are available to *C. nubeculosus* [133, 164].

Taxonomic Discussion

Culicoides nubeculosus was originally placed within *Ceratopogon* [165]. Goetghebuer (1912) described a specimen as from Belgium as *C. puncticollis*, however this name was already occupied and so Goetghebuer (1920) later changed the name to *C. punctacticollis*. These specimens were deemed to be synonyms of *C. nubeculosus* by Goetghebuer (1933). Additional descriptions of adult *C. nubeculosus* can be found in Edwards (1939), Downes (1950), Campbell and Clinton (1960), Kremer (1965), Orszagh (1980), and Glukhova (1989). The larvae and pupae

of *C. nubeculosus* were first described in Medwedewa (1927) and redescriptions are available in Mayer (1934), Kettle and Lawson (1952), Dzhafarov (1964) and Kremer (1966).

[166]

***Culicoides occidentalis* Wirth and Jones**

Culicoides (*Monoculicoides*) *variipennis occidentalis* Wirth and Jones, 1957: 21 (as subspecies). Type locality: Borax Lake, California, USA, July 1948, reared from lake margin. Holotype ♂ slide (USNM), type number 63250; Allotype ♀ pinned (USNM), same collection data; Paratype 29 ♂, 60 ♀, many L, many P slides and pinned (CAN, CDC, CIS, USNM), same collection data.

Culicoides occidentalis: Downes, 1978:63 (in part).

Culicoides occidentalis: Holbrook, 2000:71 (species designation).

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with prominent black spots on a uniformly grey scutum, caudal margin of S9 straight, and with aedeagus bare; female: only North American species of *C*. (*Monoculicoides*) with prominent black spots on a uniformly grey scutum, wing length > 1.5 mm, and with C-shaped spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 18-29 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process with narrow at base, tapering to apex; fused parameres with long, stout base, apices narrowly separated; aedeagus triangular, without spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 5-8; palpus with third segment extremly wide with large pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 14-19; spermatheca ovoid, long, C-shaped, with spermathecal ring absent.

DISTRIBUTION: *Culicoides occidentalis* is distributed in western North America known from British Columbia, Canada to northern California, east to Utah, south to Baja California, Mexico and western Texas [17, 19, 25, 167-169]. There also appears to be an extant population living in the soda lakes east of Mexico City [170].

Bionomics

Adult Habitat and Seasonality

Culicoides occidentalis adults can be collected from May to October with peak emergence occurring in the late summer in its southern distribution. [171, 172]. Adults of this species can also be collected year-round in areas with mild winter months such as southern California [173]. Nelson & Bellamy (1971) measured the flight activity of *C. occidentalis* in California and collected the greatest numbers near dusk and dawn as well as during moonlight hours. This study did not differentiate between *C. occidentalis* and *C. sonorensis* and so this study likely represents data from both species. While collecting in British Columbia, McMullen (1978) reported *C. occidentalis* collecting adults specimens from 280 – 1800 m elevation. Immature specimens from California and Mexico have been collected at elevations approaching 2000 m [170, 174].

Immature Habitat

This species can be found in highly alkaline and saline environments commonly associated with soda lakes [19, 25, 175]. Soil chemistry analyses of the larval habitat of *C. occidentalis* found high levels of dissolved salts, boron, chloride, and potassium [176, 177]. There is some overlap in the habitable range of *C. occidentalis* and *C. sonorensis*, though these sympatric habitats often align more closely with what would be considered more stereotypically *C. occidentalis* habitat [19, 25, 177]. *Culicoides occidentalis* pupae have also been collected from relatively harsh environments such as Mono Lake, California and Lake Tecuitlapa, Mexico [170, 174]. These

habitats are roughly 2-3 times as salty as the ocean and have a pH of 9-10 (the pH of household glass cleaner). Living in these harsh environments has been proposed as a means of protecting them from being parasitized by mermithids [178, 179]. When reared in the laboratory, *C. occidentalis* was found to be a good host for *Heleidomermis magnapapula*, a common *Culicoides* parasite, indicating that is absence from wild populations of *C. occidentalis* is likely due to the saline habitats [178]. Similarly, the bacterium *Bacillus thuringiensis israelensis* (Bti) was tested for efficacy against the larvae of *C. occidentalis*, but was found to be an ineffective biological control agent [171, 180].

Feeding

Blood meal analyses of *C. occidentalis* collected in California showed an incredibly diverse host breadth [181]. This species was found to have fed on cattle, deer, equines, sheep, pigs, rabbits, and occasionally emu, with the majority of the blood meals being from the large ungulates.

Mating behavior

The males of *C. occidentalis* have been observed swarming above salt marshes (the larval habitat) in southern California [182]. Stenogamy has been reported occasionally, but it does not seem to be present to the extent it is in *C. sonorensis* [167, 183]. In laboratory experiments, Velten and Mullens (1997) showed that *C. occidentalis* and *C. sonorensis* will hybridize and can produce viable offspring for at least six generations. This lack of post-zygotic reproductive isolation indicates that there must be pre-zygotic isolation barriers between these species. The differences in mating behavior has been suggested as the mechanism by which this separation is maintained [167, 182]. Though rare, Shults *et al*. (2021) showed that hybridization between these two species does occur in nature, thus mating behavior alone is not enough to prevent hybridization. Crosses between ♂ *C. sonorensis* and ♀ *C. occidentalis* yielded an egg hatch rate

of nearly 75.0%, whereas the reciprocal cross only yielded a 7.0% hatch rate. Asymmetrical hybridization could denote a recent speciation event and this directional loss of fertility is often associated with cytoplasmic incompatibility [184, 185].

Development

Dyce (1969) found a link between parity level and abdominal pigmentation of the sternite, providing an easy way to distinguish parous and nulliparous females Braverman 2009; Linley 1984. Females with a burgundy-red pigment were found to have undergone at least one gonotrophic cycle while females with undeveloped ovaries lacked this pigmentation. A notable exception to this rule was *C. occidentalis*. Individuals from Bakersfield, California were strongly pigmented (and indication of being parous), though upon dissection, all were found to be nulliparous. When collected directly from the larval habitat, Smith and Mullens (2003) found very few darkly pigmented females and so this pigmentation may just be associated with the age of the individuals [186].

Vector status

Culicoides occidentalis was determined to be a poor vector of BTV with less than 1.0% of the individuals tested being infected [187]. This highlights the usefulness of proper species identification as to not artificially lower seroprevalence data.

Molecular data

COI, COII, CAD, and TPI gene sequences are available for *C. occidentalis* [6, 188]. The COI gene has been found to separate this species from the other members of the *C. variipennis* complex; however, Shults *et al.* (2021) showed that this gene is also highly divergent in various populations of *C. occidentalis*. Sequences from individuals in northern California (Borax lake)

were just as divergent from the other populations of *C. occidentalis* as they were to *C. albertensis*, *C. sonorensis*, and *C. variipennis*.

Taxonomic Discussion

This species was first described as a subspecies of *C. variipennis* by Wirth and Jones (1957), and was placed into the *C. variipennis* complex. Jorgensen (1969) proposed that *C. occidentalis* could be an independent species as it occurred sympatrically with *C. variipennis*; however, the specimens collected by this author likely included *C. sonorensis*. Downes (1978) supported the notion of Jorgensen (1969) that the uniformity of the morphological characters seen in specimens of *C. variipennis*, even when in sympatry with other members of the *C. variipennis* complex, warranted its species status. He resurrected *C. variipennis* to a full species but also raised *C. occidentalis* to species status while designating the remaining members of the *C. variipennis* complex as nominotypical forms of *C. occidentalis*. Holbrook *et al*. (2000) was the first to recognize what we consider to be *C. occidentalis* based mainly on electrophoretic data. The pupae of *C. occidentalis* were described by Shults and Borkent (2018) and the larvae were described (as *C. variipennis*, in part) by Murphree and Mullens (1991).

Atchley (1967) examined specimens from a salt lake near Loving, New Mexico and identified these individuals as *C. sonorensis*. Holbrook *et al.* (2000) also studied these specimens and identified them as *C. occidentalis.* In our reexamination of this material, we found that the males had 3-6 spicules at the distal end of the aedeagus. This is far too few to be the typical *C. sonorensis* (30-45) but it is also not the typical character state for *C. occidentalis* either. To further confound the situation, specimens collected from a salt lake in Roosevelt County (the adjacent county) are easily identifiable as *C. occidentalis* and there are numerous stereotypical *C. sonorensis* collected from both areas. Having spicules only at the distal end of the aedeagus is

the character state found in the hybrids of these two species [183]. It is possible that this series represent hybrid offspring especially as they were all collected from the same location on the same day.

[179, 189]

Culicoides pachynonus n. sp. Shults and Borkent

Culicoides (*Monoculicoides*) *pachynonus* Shults and Borkent. Type locality: Addis Ababa, Ethiopia. Holotype ♂ slide (CNCI), labeled as "Addis Ababa, VHL house, 2,300 ft elev., 22-23.VIII.1974, light trap, VHL." **NEW SPECIES**.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with an extremely wide base of the apicolateral process and with gonostylus gradually tapering distally.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum dark brown with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 19 pleural setae; epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with extremely wide at base, tapering to apex; fused parameres with long, slender base, apices narrowly separated; aedeagus triangular, with numerous spicules; gonostylus gradually tapering distally. *Female*, unknown.

DISTRIBUTION: *Culicoides pachynonus* is known only from Addis Ababa, Ethiopia.

Bionomics

Adult Habitat and Seasonality

The ecoregion of Ethiopia where C. *pachynonus* occurs is montane grasslands and shrublands [16]. No other biological data exists for this species.

Little else is known about this species.

Taxonomic Discussion

Culicoides pachynonus is almost identical to *C. cornutus* but is much darker and with wider apicolateral processes. It is clearly a member of the subgenus *C*. (*Monoculicoides*) due to the presence of a dark spot near the arculus, fused parameres, and bifurcate aedeagus.

Culicoides parroti **Keiffer**

Culicoides parroti Kieffer, 1922:502. Type locality: Biskra, Algeria. Holotype ♀ (location unknown), "all captured on donkeys, by M. Parrot, of the Institut Pasteur, to whom I dedicate this species: Biskra, 16.V.1922 and Old Biskra road, 23.V.1922."
Culicoides (*Monoculicoides*) *parroti*: Khalaf 1954:40.

DIAGNOSIS: Male and female: only species of *C*. (*Monoculicoides*) with a grey scutum containing distinct black spots laterally and medial black spots that coalesce forming a continuous pattern. In addition, male: only species of *C*. (*Monoculicoides*) with wings lacking a pattern of pigmentation and lacking distinct posterior projection medial to base of apicolateral process; female: only species of *C*. (*Monoculicoides*) with an amorphous spermatheca slightly folded onto itself and with eyes bare.

DESCRIPTION: *Male*, **Head**: eyes separated by 2 ommatidia. **Thorax:** scutum grey with lateral black spots, medial spots touching forming a continuous pattern; wings wings without pattern. **Abdomen:** abdominal segment 1 with 8-12 pleural setae; epandrium with strongly tapering posteriorly, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex, short; fused parameres with thick medially, short, stout base, apices widely separated; aedeagus triangular, without spicules; gonostylus gradually tapering to midpoint. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow with small pit; mandibular teeth well

developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 5-8; spermatheca amorphous, slightly folded onto itself, with spermathecal ring present.

DISTRIBUTION: *Culicoides parroti* is a broadly distributed Palearctic species known from the United Kingdom east to southern European Russia, south to Morocco, Tunisia, Turkey, Iran, and Tajikistan [58, 83, 84, 86, 131, 135, 139, 164, 190-214]. It is a generally more southern species, with no records from Scandinavia or northern Russian. Glukhova (1989) reported that this species is normally collected between 400 – 1200 m in the Transcaucus region with a record of it being collected as high as 1900 m near the Kugitangtau mountains in Tajikistan.

Bionomics

Adult Habitat and Seasonality

The range of *C. parroti* is primarily distributed across Mediterranean forests, temperate broadleaf and mixed forests, and temperate grasslands, savannas and shrublands [16]. This species is often found on livestock farms occupied by sheep, pigs, cows, goats, or horses [86, 135, 202, 211], and is collected primarily from May to September with peak emergence times in June and July [193, 204, 209, 215].

Immature Habitat

The pupae of *C. parroti* have been collected from mud rich in organic material, alongside other members of the subgenus *C*. (*Monoculicoides*); *C. nubeculosus* and *C. stigma* [164, 203]. Pupae have also been collected from muddy pools with little vegetation [85] and from large expanses of open mud and moist soil in woodlands [134, 209]. Perrin *et al*. (2006) lists *C. parroti* in a group of halophilic species based on a multiple correspondence analysis of molecular, ecological, and morphological data. Though saline environments are likely not the primary habitat, *C. stigma*

and *C. nubeculosus* have occasionally been collected from salt marshes, suggesting that this may also be the case for *C. parroti* [15].

Feeding

Specimens used in the original description in Kieffer (1922) were collected from donkeys. Kieffer (1923) noted that this species would also feed on humans. *Culicoides parroti* has been aspirated directly from sheep and horses [197, 215, 216] and blood-meal analyses showed that this species feeds on sheep and deer [217, 218]. This species has been found in association with zoos in the UK and Spain and is likely to feed on a variety of wildlife including zebra and giraffe [194, 204, 219]. In Spain, *C. parroti* was the second most numerous species feeding on sheep [215]. Host-seeking began approximately 90 minutes before sunset, with little to no feeding activity reported after dark. The host attack rate for this species was estimated at almost 1 per minute, highlighting the immense burden this species can place on livestock.

Mating behavior

Gerry *et al*. (2009) showed a discrepancy in the sex ratio of specimens collected with and without the use of CO_2. Many more males were collected from un-baited UV traps suggesting that this species may not mate at the host.

Development

In Spain, larvae of *C. parroti* were observed being parasitized by the mermithid nematode *Heleidomermis cataloniensis* [209].

Vector status

This species is a suspected vector of BTV, though there is no evidence that it actually plays a role in transmission. This status is based solely on its close association with susceptible hosts and its relation to *C. sonorensis*. During an outbreak of Schmallenberg disease in France, four *C.*

parroti were tested for the virus but were found to be negative [131, 155]. This species has been found infected with *Onchocerca cervicalis*, a parasitic round worm, and is likely a vector to horses [220].

Molecular data

COI and ITS-1 sequences have been obtained from specimens collected in France, Morocco, and Slovakia [86, 164, 210, 221].

Taxonomic Discussion

After describing the females of *C. parroti*, Kieffer (1923) described the males of this species. Redescriptions of both sexes were done by Edwards (1939) from specimens collected in the UK and Turkey. Additional descriptions of the adults can be found in Campbell and Pelham-Clinton (1960), Coluzzi and Kremer (1964), Dzharforov (1964), Kremer (1965), Orszagh (1983), and Glukhova (1989). All of these are in general agreement with our assessment of *C. parroti*. Kettle and Lawson (1952) described the pupae of *C. parroti* but do not list from what habitat these specimens were collected. As others have noted that this species can be collected in the same habitat as *C. stigma* and *C. nubeculosus*, we would assume the habitat listed for these two species are also the source of the *C. parroti* collected. Khalaf (1954) placed this species in the *nubeculosus* group of *C*. (*Monoculicoides*). This species is included in the complete version of the online interactive identification key for female *Culicoides* from the west palearctic region (IIKC) [222, 223].

The description of the Chinese species *C. combinothecus* is very similar to *C. parroti*. The differences listed by Yu (1986) are the shape of the spermatheca as well as the presence of ocular setae which were stated as not being found in *C. parroti*. However, Yu *et al*. (2005), states that these ocular setae are also present in *C. parroti* from China. Additionally, the shape of the

spermatheca of *C. combinothecus* is very similar to the spermatheca of *C. parroti* when viewed dorsally (see the photo in Gonzalez and Goldarazena (2011) [137]). Of the *C. parroti* material we examined, all from the western Palaearctic, none were found with ocular setae. It may be that the absence or presence of ocular setae is an expression of geographical variation within *C. parroti* but this seems unlikely considering this has not been reported in any other *C.* (*Monoculicoides*). It is likely that *C. combinothecus* is a valid species occurring only in China. In this case, the *C. parroti* described with ocular setae from China are misidentified and are *C. combinothecus*.

***Culicoides puncticollis* (Becker)**

Ceratopogon pulicaris forma algecirensis Strobl, 1900:170. Syntype ♂, Type locality: Spain (?lost); Szadziewski 1986:69 neotype ♀ pinned, Spain (ZMHB). Name suppressed by ICZN Opinion (1989) [224].
Ceratopogon puncticollis Becker, 1903:75. Alexandria, Egypt. Holotype ♀ (ZMHB), collected in May.
Culicoides algecirensis: Kieffer 1919:39.
Culicoides impressus Kieffer, 1918:47. Syntypes ♀, ♂, Tunisia (?HNHM).
Culicoides distigma Kieffer, 1922:502. Type(s) ♀, Algeria (unknown).
Culicoides donatieni Kieffer, 1922:504. Type(s) ♀, Algeria (unknown).
Culicoides sciniphes Kieffer, 1925:261. Type(s) ♀, Egypt (unknown).
Culicoides bipunctatus Vimmer, 1932:133. Type(s), sex unknown, Israel (unknown, ?NMPC).
Culicoides griseovittatus Vimmer, 1932:133. Type(s), sex unknown, Israel (unknown, ?NMPC).
Culicoides tripunctatus Vimmer, 1932:137. Syntypes ♀, Type locality: Israel (NMPC).
Culicoides flavitarsis Vimmer, 1932:137. Type(s), sex unknown, Israel (unknown, ?NMPC).
Culicoides wenigi Vimmer, 1932:137. ?Holotype ♀, Israel (NMPC).
Culicoides luteosignatus Vimmer, 1932:140. Type(s) ♀, Israel (unknown, ?NMPC).
Culicoides vavrai Vimmer, 1932:140. ?Holotype ♀, Israel (NMPC).
Culicoides bivittatus Vimmer, 1932: species never published but was deposited into the NMPC, Kremer 1981:2.
Culicoides (*Monoculicoides*) *puncticollis*: Khalaf, 1954:40.

DIAGNOSIS: Male and female: only species of *C.* (*Monoculicoides*) with diffused black spots on scutum. In addition: male: only species of *C.* (*Monoculicoides*) without spicules on aedeagus

and S9 with small, undefined medial cleft; female: only species of *C*. (*Monoculicoides*) with a long, straight, sclerotized spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum light grey with small black dots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 10-27 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process with narrow at base, tapering to apex; fused parameres with thick medially, short, stout base, apices narrowly separated; aedeagus triangular, without spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 4-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 16-18; spermatheca ovoid, long, with spermathecal ring absent.

DISTRIBUTION: *Culicoides puncticollis* is known from the United Kingdom to Morocco and Algeria, east to Kazakstan and Pakistan. Also known from southwestern Siberia, and northern and central China [58, 71, 83, 84, 136, 141, 200, 203, 225-237].

Bionomics

Adult Habitat and Seasonality

Culicoides puncticollis is broadly distributed across the Mediterranean forests, woodlands and scrubland of Europe and Northern Africa; the steppes of eastern Europe, Central Asia, and Russia; and the deserts & xeric shrublands of the Middle East [16]. Both Dzhafarov (1964) and Glukhova (1971) considered this species to occur only in arid steppes and semidesert regions; however, reports from the UK, Denmark, and other temperate areas contradict this [135, 238].

These differences in habitat type could be an indication of more than one species and this idea is discussed further in the taxonomic discussion. *Culicoides puncticollis* adults are active from May to September in its northern distribution [135], though in the Mediterranean and more arid regions, this species can be found year-round [239]. Peak emergence times appear to be July, August, or September depending on the location [135, 228, 234].

Immature Habitat

The immature stages of *C. puncticollis* are often associated with livestock operations and breed readily in mud rich in animal dung and other organic material [134, 228, 230, 239]. In more natural areas, *C. puncticollis* larvae live in shallow reservoirs with silty soil, silted banks of lakes, ponds, rivers, streams, as well as man-made irrigation canals [58, 240]. In studying larval habitats of the Pontic steppe of eastern Europe, Dubrovskaya (1980) found that larvae of this species prefer relatively warm waters with little vegetation, heated by direct sunlight. Though *C. puncticollis* larvae were collected occasionally alongside *C. riethi* in saltwater marches, this was rare.

Feeding

Culicoides puncticollis females have been reported feeding on a variety of mammals including, horses, donkeys, sheep, cattle, and humans [214, 229, 233, 237]. Feeding occurs mainly at dusk and host seeking tapers off into the night, though strong moonlight can increase the time spent actively feeding [241]. In western Siberia, female *C. puncticollis* severely attack live stock from August to September [234].

Mating behavior

Male swarms can be found at or near the host and coupling occurs on the animal, though copulation without swarming has also been observed [242]. The only other species of *C*. (*Monoculicoides*) where this behavior occurs in *C*. *sonorensis* and *C. nubeculosus*.

Development

Culicoides puncticollis females are not autogenous and must take a blood meal for the eggs to develop [243]. Maturation of the eggs occurs between 3-4 days, and once the eggs are laid, larvae hatch within the first three days [13, 244]. Glukhova (1967) successfully reared *C. puncticollis* larvae on the medium used by Jones (1957) as well as an Azotobacter film, with direct observations of feeding on this material. Interestingly, Glukhova (1967) also sampled the gut contents of larval *C. puncticollis* and found the bacterial composition within the midgut was the same as what was found in the silt where they live; however, at a much higher concentration.

Vector status

The association with livestock appears to be the only evidence for the adult females being potential vectors of disease causing pathogens. In testing for BTV in Israel, all *C. puncticollis* tested were negative [245]. In a laboratory trial, AHS, Akbane virus, and BTV failed to multiply in *C. puncticollis* after oral ingestion and all were inactivated by four days post infection [246].

Molecular data

Sequences of the COI, 28S, ITS, and CAD genes are available in Genbank [238, 247]. Nielsen *et al*. (2014) recorded *C. puncticollis* for the first time in Denmark and this represents by far the most northern distribution of this species. Slama *et al*. (2014) compared the COI gene of individuals collected in Tunisia to those collected in Denmark and found some sequence divergence, though it remains to be seen if this is merely geographic variation.

Taxonomic Discussion

This species was first described from specimens collected in Spain (which are now lost) [228], and Strobl (1900) considered it to be a form of *Ceratopogon pulicaris* [*Culicoides pulicaris*]. In 1903, Becker described *Ceratopogon puncticollis* from specimens collected in Egypt. Strobl (1906) raised *Ceratopogon algecirensis* to species status and Kieffer (1919) later moved it to *Culicoides*. Edwards (1939) then synonymized *Culicoides algecirensis* with *Culicoides puncticollis* though technically the species name *algecirensis* should have taken precedence. This name was suppressed however by ICZN opinion as proposed by John Boorman [1]. A neotype of *C. algecirensis* was designated by Szadziewski (1986). Between 1918-1932, Vimmer described 11 species from the Middle East an northern Africa that have all since been synonymized with *C. puncticollis*. First Goetghebuer (1934) combined the junior synonyms *C. bipunctatus* with *C. flavitarsis*; afterwhich Edwards *et al*. (1939) synonymized *C. impressus*, *C. algecirensis*, *C. donatieni*, *C. sciniphes,* and *C. flavitarsis* with *C. puncticollis*. These authors also list *C. vavrai* as a synonym but denote it with a question mark. Later, Kremer *et al*. (1981) synonymized *C. tripunctatus, C. vavrai,* and *C. wenigi*, and finally Szadziewski (1984) synonymized *C. distigma*, *C. griseovitatus*, and *C. luteosignatus*. Additional descriptions of the adults can be found in Gutsevich (1953), Khalaf (1957), Clastrier (1957), Gutsevich (1960), Dzhafarov (1964), Navai and Mesghali (1968), Navai (1970), and Boorman (1976). The pupae are described in Kieffer (1923) [as *C. donatieni*] and Dzarfarov (1964); and the larvae are described in Glukhova (1968, 1977, 1979), Gutsevich and Glukhova (1970) and Chaker (1983).

It has been proposed that *C. riethi* and *C. puncticollis* represent the same species as northern and southern forms [248, 249]; however, there are several morphological characters that separate them [123] as well as differences in the larval habitat [240]. Comprehensive studies further investigating *C. puncticollis* instead considered it to be a valid but strongly variable

species [123, 214]. This morphological variability and broad distribution across multiple biome may be an indication that there are multiple species within *C. puncticollis*. Glukhova (1971) compared the amount of variation seen in this species with that observed for the *C. variipennis* complex. Thus far, the molecular data available for *C. puncticollis* provides good evidence that this species is separate from that of *C. riethi* and *C. nubeculosus*; however these data do not exclude the possibility of a cryptic species. The records that we consider to warrant further investigation based on the distribution of *C. puncticollis* are specimens from the UK and Denmark.

Culicoides riethi **Kieffer**

Culicoides riethi Kieffer, 1914:237. Type locality: Westfalen, Sassendorf and Salzkotten, Germany. Holotype ♂ (location unknown); Allotype ♀ (location unknown), same locality data.
Culicoides cordatus Kieffer, 1921a:114 (1921b:275). Type locality: Libau [Liepāja], Latvia, no holotype designated.
Culicoides crassiforceps Kieffer, 1924:15. Type locality: Holstein, Germany, no holotype designated.
Culicoides gigas Root and Hoffman, 1937:172. Type locality: Fort a la Come, Canada. Holotype ♀ pinned (CNCI), labeled as "July 17, 1925, Kenneth M. King."
Culicoides (*Monoculicoides*) *riethi*: Khalaf 1954:40.

DIAGNOSIS: Male and female: only species of *C*. (*Monoculicoides*) with diffused black spots on scutum. In addition: male: only species of *C*. (*Monoculicoides*) without spicules on aedeagus and S9 with large, well-defined medial cleft; female: only species of *C*. (*Monoculicoides*) with ovoid spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 17-22 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight;

apicolateral process with wide at base, tapering to apex; fused parameres with thick medially, long, stout base, apices narrowly separated; aedeagus triangular, without spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 5-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 15-18; spermatheca ovoid, with spermathecal ring absent.

DISTRIBUTION: *Culicoides riethi* has a Holarctic distribution. In the Palaearctic, it occurs from the United Kingdom to Spain, east to the Ural mountains in Russia and south to northern Iran and Egypt as well as from the eastern Eurasian steppe, Kazakhstan, southern Siberia, Mongolia, and northern China. In the Nearctic, *C. riethi* occurs from Alaska to British Columbia, east to Manitoba and south to Nebraska [21, 26, 55, 58, 64, 72, 77, 81, 84, 136, 139, 141, 199, 205, 214, 231, 238, 250-265].

Bionomics

Adult Habitat and Seasonality

Culicoides riethi adults are active from May to September [135, 214, 258, 261, 266] and evidence from Germany suggests that the minimum temperature required for flight activity is approximately 13°C [267]. In Alberta, Canada, Walgama and Lysyk (2019) collected adults from light traps between 10-30°C from dusk until dawn. Dzhafarov (1964) collected specimens from the Caucasus region, mainly in open meadows and bog areas, and found that *C. riethi* occurs sympatrically with both *C. puncticollis* and *C. nubeculosus*. Females are most active from dusk until dawn but can occasionally be collected during the day from CDC light traps [264, 268], the males however, are almost never collected during the day and are most active just before dusk

[214]. Adults can be found resting on the lower part of trees as well as bushes, reeds, or grasses near the larval habitat [214].

Immature Habitat

Throughout much of Europe, *C. riethi* is considered to be a halophilic species and the immatures can be found at muddy margins of salt springs, salt marshes, and tidal pools [248, 257, 269-272]. In Russia, it has also been collected from mud surrounding highly saline lakes [273, 274]. While common in these types of habitats, Boorman (1986) stated that this species "is by no means confined to them." In both the Palearctic and Nearctic, immature *C. riethi* can be found at the margin of alkaline pools and sloughs [21, 264], and in North America, it occurs in the same habitat as *C. albertensis* and *C. shemanchuki* [64]. In the Republic of Kalmykia, Russia, almost all bodies of water served as breeding sites for *C. riethi*. The most numerous accumulations were found in puddles and small ditches of a floodplain [275]. In both Turkey and Tunisia, this species is reported to live in mud rich in dung near water reservoirs [276, 277]; however, this may be a misidentification of *C. puncticollis*.

Feeding

Direct aspirations and blood meal analysis show that adult female *C. riethi* feed on livestock such as cattle, goats, and horses [161, 260] and have also been reported to occasionally bite humans [21, 58, 264]. Host seeking usually begins at dusk and attacks are known to take place throughout the night [214, 264, 275].

Mating behavior

Mating swarms near the larval habitat have been observed [253, 278]. Males will begin swarming approximately 1.5-2.0 hours before dusk (when the females are most active) though this shortens to about 30 minutes before dusk towards the end of the season [214]. The males use

light colored objects as mating markers and form 0.6-0.9 m columns roughly 0.6 - 1.2 m from the ground [147]. Downes (1955) was able to induce swarming by placing a white sheet on the ground and both Dzhafarov (1964) and Dubrovskaya (1974) report this species swarming over the heads of humans. The swarms will shift to ensure the males were facing into the wind but mating itself appears to occur downwind after coupling [147]. This species will also mate in confined spaces which allowed for its colonization [148].

Development

It has been proposed that this species has at least three to four generations per year [214], though this likely increases in areas where adults can be collected almost year-round [255]. Autogeny has been confirmed in many natural populations [148, 214, 243, 275, 279], although a blood meal is needed to produce a second batch of eggs [148, 255]. Oviposition flights take place at sunset, and eggs are laid in the soil at the margin of the larval habitat [21], a single female can lay between 90-240 eggs [214]. Under laboratory conditions, pupation began around 14 days after eggs were laid, but peaked between 18-21 days. This is a longer development time than *C. sonorensis* [280], but *C. riethi* is a larger species. Adults emerged from the pupae after 2-3 days and females were able to lay a batch of eggs within the first three days of emergence [148, 281]. Females are reluctant to feed in the laboratory as so this species was maintained in colony autogenously for approximately two years before the colony was discontinued [148]. Mukanov (1978) notes that as the soil temperature reached 10°C in late September, adults and pupae can no longer be collected while the larvae can be found into the late autumn months. This is an indication that *C. riethi* overwinters in the larval stage. A survey conducted in the Sichuan provence of China found female adult midges being parasitized by an ectoparasitic mite, an endoparasitic ciliate (*Blantidium* sp.), and a nematode belonging to the Mermithidae family

[255]. Some of the females found with mermithid nematodes experienced ovarian degeneration and sterilization.

Vector status

Both BTV and EHDV were able to replicate in female *C. riethi* after intrathoracic inoculation; however, an inability to induce blood feeding prevented these studies from investigating oral transmission of the virus [157, 282]. One positive instance of BTV was found in Italy from a pooled sample containing *C. nubeculosus*, *C. puncticollis*, and *C. riethi* [283]. There is very little evidence that this species is responsible for pathogen transmission though it has been implicated as a potential vector of filarial worms [161].

Molecular data

COI sequences if *C. riethi* have been obtained from several studies [6, 210, 260, 263] and are available in GenBank.

Taxonomic Discussion

The male and female adults of *C. riethi* were first described by Kieffer (1914). Kieffer then went on to describe two closely related taxa, *Culicoides cordatus* and *Culicoides crassiforceps*, which Edwards (1939) recognized as synonyms of *C. riethi*. Edwards (1939) also lists C. *pullatus* as a synonym of *C. riethi*; however, this name was later determined to be a synonym of *C. pulicaris* instead by Kieffer (1915). Khalaf (1954) designated *C. riethi* as a member of the subgenus *C.* (*Monoculicoides*). Remm (1988) lists *C. osakensis* as a synonym of *C. riethi*; however, based on the distribution and original description of *C. osakensis*, we agree with Vargas (1949) who first placed it as a synonym of *C. homotomus* that it belongs there. Lastly, Grogan and Lysyk (2015), comparing material from the Nearctic and Palearctic Regions, concluded that there were no discernable morphological differences between adults of *C. gigas* and *C. riethi*. Thus, they

designated *C. gigas* as a junior synonym. In the original description of *C. gigas*, Root and Hoffman (1937) report this species as having uniform yellowish brown legs and lacked a distinctive scutal pattern. Grogan and Lysyk (2015) note that these character states are inconsistent with the Nearctic specimens they studied. We examined the holotype of *C. gigas*, and this specimen is sun-bleached which is likely why Root and Hoffman (1937) described it as they did. Though damaged, there is still evidence of a banding pattern on the legs, which Root and Hoffman (1937) reported as being absent, as well as the typical *C. riethi* pattern on the scutum. Consistent with the morphological evidence, the immatures of both *C. riethi* and *C. gigas* can be collected in highly saline or alkaline habitats, both females possess autogeny, and both occur in similar bioregions of Europe/Asia and North America. From the lack of morphological differences in the adults and pupae (mentioned in a letter from Willis Wirth, and reaffirmed here) [64], as well as similarity in habitat and biology, we agree with Grogan and Lysyk (2015) that *C. gigas* is in fact *C. riethi*.

Additional descriptions of the adults of *C. riethi* can be found in Gutsevich (1960, 173), Campbell (1960), Dzhafarov (1964), Kremer (1965), and Glukhova (1971, 1979). All of these are in general agreement with our descriptions of this species. Several authors have proposed that *C. riethi* and *C. puncticollis* represent a northern and southern form of the same species [214, 248, 249, 284, 285]; however, Glukhova (1979) conducted a detailed analysis of material from many different regions and provided both male and female characters that distinguish them. *Culicoides riethi* females have a much longer spermathecal duct and *C. riethi* males possess a V-shape notch on the 9th abdominal segment that is not present in *C. puncticollis*. Glukhova (1979) also mentions that much of the uncertainty around these two species stemmed from only using

wing and scutal patterns for identification and showed these characters to vary widely based on seasonality for *C. nubeculosus*, *C. puncticollis*, and *C. riethi*.

Kettle and Lawson (1952) provided the first detailed descriptions of the immatures of *C. riethi* including keys to separate this species from *C. nubeculosus*, *C. stigma*, and *C. parroti*. Prior to this, Rieth (1915), Thienemann (1928) (as both *C. riethi* and *C. crassiforceps*), Mayer (1934) (as both *C. riethi* and *C. crassiforceps*), and Lenz (1934) provide descriptive notes on the pupae of *C. riethi* but do not thoroughly describe this life stage. Rieth (1915), Thienemann (1928), and Mayer (1934) provide notes for the larvae of *C. riethi* as well. The pupae of *C. riethi* were subsequently described in Dzhafarov (1961 & 1964), Damian-Georgescu and Spătaru (1971), and Glukhova (1989); the larvae were described in Dzhafarov (1961, 1964), Damian-Georgescu and Spătaru (1971), Glukhova (1968, 1977, 1979), and Knoz (1980); and the eggs were described in Dzhafarov (1961, 1964) and Kremer (1966).

Throughout most of Europe *C. riethi* is listed as a common species but it is not reported in great numbers [258, 262, 286]. In the Eurasian steppe however, *C. riethi* is listed as one of the most prevalent species collected [55, 214, 250, 287, 288]. *Culicoides riethi* in Morocco were reexamined by Kremer (1971) and were found to instead be *C. punticollis* and this could also be the case for the reports of *C. riethi* from Tunisia [86, 276]. As we were unable to obtain specimens from this region, we were unable to confirm this. *Culicoides riethi* have also been collected in Egypt and in examining these specimens, we agree with the species identification. As *C. riethi* does not occur in more arid regions, we believe that its distribution is likely restricted to the north near the Mediterranean sea and Nile river estuary. This populations may be connected to the rest of its distribution by the habitat around the Mediterranean Sea in Israel, Lebanon, and Syria.

Culicoides shemanchuki Grogan and Lysyk

Culicoides (*Monoculicoides*) *shemanchuki* Grogan and Lysyk, 2015:3. Type locality: Fort Macleod, Alberta, Canada. Holotype ♂ slide (CNCI), labelled as "*Culicoides peiganus*, Macleod, Alta., 20 June 1955, J.A. Downes, 194/6/14"; Allotype ♀ slide (CNCI), same locality data; Paratypes 15 ♂, 18 ♀ slides (CNCI), same locality data; Paratypes 11 ♂, 9 ♀ slides, each with associated pupal exuviae (USNM), Pierce Co., North Dakota, USA, Pleasant Lake, June 1969, alkali lake.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with the epandrium strongly tapering posteriorly; female: only species of *C*. (*Monoculicoides*) with teeth present, but reduced in number (3-5).

DESCRIPTION: *Male*, **Head**: eyes separated by 2 ommatidia. **Thorax:** scutum brown with 2 light brown stripes; wings with faint pattern of pigmentation. **Abdomen:** abdominal segment 1 with 6-8 pleural setae; epandrium with strongly tapering posteriorly, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex; fused parameres with thick medially, short, stout base, apices narrowly separated; aedeagus cylindrical, without spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment wide with small pit; mandibular teeth reduced. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 5-8; spermatheca ovoid, with spermathecal ring absent.

DISTRIBUTION: *Culicoides shemanchuki* is known from central Alberta, Canada to North Dakota, USA [64].

Bionomics

Adult Habitat and Seasonality

Culicoides shemanchuki is known only to live in the northern shortgrass prairies of North America and has been collected from June to September [16, 64].

Immature Habitat

Downes (1958) that habitat where this species (as *Culicoides* sp. nov.) was first collected as pupae from mud around alkaline prairie sloughs. He also notes that in these habitats, the larval density can be quite high.

Feeding

Downes (1971) reported that this species (as *Culicoides* sp. nov.) had weakly sclerotized mouthparts and a shorten proboscis. The reduction of mandibular teeth of female adult *C. shemanchuki* indicates this species does not feed or at least does not take a blood-meal [289].

Mating behavior

No swarming has been reported in this species [289]. Instead, both male and female C. *shemanchuki* were observed running around randomly on top of the mud's surface and mating was initiated on contact. This type of mating behavior has not been recorded in any other *Culicoides* species. Other species of *C*. (*Monoculicoides*) are known to mate without the need of forming swarms; however, swarming is also known in these species or they mate on or near the host [147, 290]. Downes (1958) also notes a reduction the plume of the male antennae, a possible autapomorphy, and suggests that it is non-functional in relation to mating.

Development

Downes (1971) considered this species to be autogenous as the ovaries developed 2-3 days after emergence [289]. The development of ovarian follicles before ingesting a meal has been noted in other *C*. (*Monoculicoides*) [291], and without teeth, we would assume this species is autogenous.

Vector status

As this species likely does not take a blood-meal, it would not play a role in pathogen transmission.

Molecular data

A partial sequence on of the COI gene exists for *C. shemanchuki* (Genbank assentation number KT794134.1) [6].

Taxonomic Discussion

Grogan and Lysyk (2015) noted that *C. shemanchuki* closely resembles *C. riethi*, but other than their large sizes and similar looking spermatheca, there is not much similarity. The male genitalia of *C. shemanchuki* most closely resembles that of *C. nubeculosus* with a strongly tapering epandrium, cylindrical hypandruim, and gradually tapering gonostylus. The female of *C. shemanchuki* shares a reduction in mandibular teeth, brown scutum with light brown stripes, and lack of pattern on the wing with *C. grandensis*. The pupa of *C. shemanchuki* is described in Shults and Borkent (2018).

[292]

***Culicoides sonorensis* Wirth and Jones**

Culicoides (*Monoculicoides*) *variipennis sonorensis* Wirth and Jones, 1957:18 (as subspecies). Type locality: St. David, Arizona, USA, October 1953, light trap. Holotype ♂ slide (USNM), type number 63249; Allotype ♀ slide (USNM), same collection data; Paratypes 10 ♂ slides, 47 ♀ slides and pinned) (USNM), same locality data, dates from September-October, 1953.
Culicoides occidentalis sonorensis: Downes, 1978:63
Culicoides sonorensis: Holbrook, 2000:70

DIAGNOSIS: Male: only species of *C.* (*Monoculicoides*) with numerous spicules on the aedeagus and gonostylus gradually tapering to distal end; female only species of *C.* (*Monoculicoides*) with a C-shaped spermatheca and wing length generally under 1.5 mm.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1 with 8-12 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process with narrow at base, tapering to apex; fused parameres with long, stout base, apices narrowly separated; aedeagus triangular, with numerous spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 5-8; palpus with third segment extremly wide with large pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 7-11; spermatheca ovoid, long, C-shaped, with spermathecal ring absent.

DISTRIBUTION: *Culicoides sonorensis* is distributed throughout most of the western USA and is common from Idaho to Baja California, Mexico, east to North Dakota and south to Mexico City, Mexico. It occurs occasionally in British Colombia and Alberta, Canada to Washington and Oregon, as well as Missouri to Virginia south to Louisiana and Florida [17, 19, 293-296].

Bionomics

Adult Habitat and Seasonality

In cooler climates such as Colorado and northern California, *C. sonorensis* can be collected from April to October with a peak emergence in the summer months [297-299]. In warmer climates, this species can be collected year round [6, 295, 300]. Diel activity for this species is greatest near sunset with a secondary peak at sunrise [301-303] and can be extended into the nocturnal hours during the summer months as well as moonlit nights [189, 297]. Nelson and Bellamy (1971) found that *C. sonorensis* were active at temperatures above 17°C and decreased substantially at temperatures lower than 13°C. Lillie et al. (1991) used mark-release-recapture to

determine the distance both males and females traveled from the larval habitat. This study showed the mean distance traveled by females was ~2.0 km with a maximum of 4.0 km. The distance traveled by the males was much lower, 0.8 km. Mullens (1985) showed that nightly dispersal of *C. sonorensis* from the larval habitat was based primarily on the direction of the wind.

Immature Habitat

This species is found in mud contaminated with organic material such as those of sewage effluents and other bodies of water associated with livestock [176, 177, 298-300, 304-306]. Before livestock production in North America, watering holes associated with buffalo may have served as the predominant habitat for this species [307]. *Culicoides sonorensis* larvae are most often collected at the water's edge or 7 cm below it (not found in deeper water) and the pupae are collected also at the shoreline or 7 cm above it [298, 304]. As the collection of high numbers of immatures is possible, several colony lines have been established and maintained over the years [290, 308, 309]. The microbial community and associated microorganisms found in the larval habitat were found to be important when attempting to colonize this species [280, 290], and there was no difference in the microbial flora isolated from natural breeding sites and laboratory colonies [310]. The importance of this bacterial community is likely due to the food availability as *C. sonorensis* larvae have been observed feeding on bacteriophage nematodes and other microorganisms [311]. The larvae of *C. sonorensis* is also parasitized by Mermithidae nematodes, and this has been shown in both laboratory and natural habitats [311, 312]. These parasites are absent from saline habitats and thus *C. sonorensis* is a more likely natural host than, for example, *C. occidentalis* [312].

Feeding

Bloodmeal analysis from *C. sonorensis* collected in California showed this species feeding on cattle and rabbits though occasionally a dog or horse bloodmeal was also found [313]. Hopken *et al*. (2017), sampling a much broader geographic distribution, expanded this list to include pigs, deer, donkeys, and emu. Tempelis *et al.* (1971) report that the majority of the bloodmeals tested came from cattle and rabbit while Hopken *et al*. (2017) found many more deer bloodmeals. This species can be found in abundance in traps baited with cattle or sheep [304, 314]. Both sexes are known to feed on sources of sugar, possibly from nectar [300].

Mating behavior

Mullens (1985) observed *C. sonorensis* males swarming at the larval habitat; however, Gerry and Mullens (1998) reports that this species swarms at or near the host. Males of *C. sonorensis* have also been shown to be attracted to CO_2 and this may be due to the fact that mating does occur at the host [182, 315, 316]. A majority of the males examined in Mullens (1985) were collected from CO_2-baited light traps 50-100 m away from the larval habitat (even though there were traps much closer), but it is unclear if this is evidence of male dispersal or simply due to wind. While *C. sonorensis* could have two different mating habits (i.e. mating near the larval habitat or at a host), this species ability to mate at the host appears to be unique within the *C. variipennis* complex and *C. nubeculosus*.

Development

Culicoides sonorensis larvae can occur in great numbers, especially on dairy farms [317], and this overcrowding can lead to the production of much smaller adults [306]. Additionally, climate effects adult size and a single population can experience a large amount of variation throughout the year [318]. Mullens (1987) found the female wing length in a population of *C. sonorensis* in southern California varied from 1.5-1.9 mm across the seasons. In general, *C. sonorensis* is the

smallest member of the *C. variipennis* complex; however, these studies show that environmental factors affect morphology and care should be taken when using size in species identification. In Northeastern Colorado, *C. sonorensis* populations produce seven generations per year with approximately two weeks between each [298]. In warmer climates, this increases to 9-11 generations per year with an average of 5-6 weeks in between [318].

As cooler temperatures arrive, both larval development and female clutch size are reduced [319]. This species overwinters as fourth instar larvae and they will burrow deeper into the mud during the colder months [320, 321]. This adaptation allows *C. sonorensis* to maintain populations in its northern distribution, but the adults may also possess a mechanism for overcoming sporadic cold periods. Significant mortality was observed in adults midges exposed to temperatures below -10°C; however, with an acclimation period at 5°C this species was able to withstand temperatures below freezing for a short period of time [322]. This cold shock treatment caused the production of seven "stress proteins" that may enable this enhanced survival response [323].

Vector status

Unlike in *C. occidentalis*, using abdominal pigmentation to age-grade *C. sonorensis* has been shown to be highly accurate [186, 300, 324]. Because of this, Mullens (1985) showed that females collected from light traps were at least 21 days old and had undergone 3-4 oogenesis cycles. This indicates that the lifespan of *C. sonorensis* is sufficiently long to support the intrinsic incubation of several viral pathogens. *Culicoides sonorensis* was first identified as a vector of BTV in west Texas and has been collected in abundance during many outbreaks [325, 326]. Virus isolated from field-collected individuals was used to infect laboratory sheep that became symptomatic [327]. The seroprevalence rate of BTV in most *C. sonorensis* populations

appears to be approximately 25% though rates as high as 50% have been reported [187]. More recently, this species has also been implicated in the transmission of EHD among white-tailed deer as EHDV was isolated from field-collected individuals [328]. However, the rarity of *C. sonorensis* in areas where EHD is prevalent may indicate that another species is likely to be the primary vector. Animal to animal transmission of BTV and EHDV via *C. sonorensis* have been demonstrated in cattle, sheep, and deer [329-332]. This appears to be the only means by which midges are naturally infected as there is no evidence of transovarial transmission [333, 334]. This also excludes the larvae from being the overwintering mechanism for BTV and EHDV in North America. A multitude of other viruses have been isolated from *C. sonorensis* including Buttonwillow, Lokern, Main Drain, and Vesicular Stomatitis virus (VSV) [335, 336]. Interestingly, venereal transmission of VSV has been shown in this species under laboratory conditions [337].

Laboratory studies have shown both infection and transmission of BTV and EHDV in *C. sonorensis* [329-331, 338-343]. These viruses are able to escape many dissemination barriers within the midges and have been isolated from various tissues including the salivary glands [344-347]. A high level of replication of BTV has also been shown [348] though this can vary based on viral strain [349] or even within individuals of the same population [350]. The use of colony data alone may not accurately represent the natural infection or transmission rate for a number of pathogens [351, 352]. Through selective breeding, Jones (1964) was able to create two colony lines, one resistant to BTV infection and another that was highly susceptible. In analyzing these two lines, a single genetic locus, later identified as glutathione S-transferase (gst-1), was identified as a potential mechanism controlling vector competence [353]. This gene was found to be highly expressed in individuals refractory for BTV [354].

While resistance to infection has been shown from a single bloodmeal, multiple feedings greatly increases the chance of infection [333]. In addition to increaseing infection rates, subsequent blood meals have the potential of introducing multiple viral strains within a single midge. This would increase the risk of reassortment and therefore has the potential of creating more variants [159, 355]. Not only is *C. sonorensis* a vector of BTV and EHDV, but also has the potential to transmit African horse sickness virus (AHSV) [356, 357], Akbane virus (AKA) [156], VSV [358-360], Oropouche virus [361], and Eubenangee virus [158]. While these other viruses do not occur within the distribution of *C. sonorensis*, its ability to harbor many different arboviruses demonstrates a high level of risks should any be introduced to North America.

Molecular data

The ease of working with *C. sonorensis* in colonies has led to an abundance of molecular information. Transcriptome data are available from several studies investigating the differential expression of genes involved in both blood feeding and vector competency [362-364]. The genome of *C. sonorensis* has also been published and is arranged in scaffolds [354]. Karotype data shows, like many other Culicomorpha [365] including Ceratopogonidae, this genome is composed of three pairs of relatively similarly sized chromosomes [366, 367]. Additionally, molecular markers have been developed to study the population structure of *C. sonorensis* [368] and a multitude of mitochondrial and nuclear genes have been sequence for this species [6, 188, 369].

Taxonomic Discussion

The adults of *C. sonorensis* are described in Wirth and Jones (1957) and Holbrook *et al*. (2000). Partial descriptions of the larva and pupa of *C. sonorensis* can be found in Wirth (1952). As he collected from the larval habitat of both *C. occidentalis* and *C. sonorensis*, these descriptions are

possibly from two species. Borkent (2012, 2014) provide partial descriptions of the pupa of *C. sonorensis* as it was used as the morphotype for the *Culicoides* pupal illustrations. The egg and larval stages of *C. sonorensis* are described in Abubekerov and Mullens (2018) and the pupa is described in detail by Shults *et al*. (2016). A key to the Nearctic pupae can be found in Shults and Borkent (2018).

Our examination of the adults of *C. sonorensis* align closely with the original descriptions in Wirth and Jones (1957, as *C. variipennis sonorensis*). In many populations throughout the southwestern USA, *C. sonorensis* is relatively small with little morphological variation between individuals. Within its most western distribution, it occurs sympatrically with *C. occidentalis*, and the male genitalia and number of plural setae on abdominal segment 1 can be used to separate these species. In areas where it occurs sympatrically with *C. albertensis* and *C. variipennis*, namely Kansas, Missouri, Oklahoma, and Arkansas, morphological separation becomes more difficult. Most of the characters that can be used to separate *C. sonorensis* and *C. variipennis*, such as the width of the third palpal segment, do not clearly separate either of these species from *C. albertensis*. Additionally, in examining the specimens identified as *C. australis*, we also see the variation described in Wirth and Jones (1957), but it remains unclear if this denotes a further species and I have conservatively considered it to be conspecific with *C. sonorensis*, as did Atchley (1967). To more fully disentangle these species will likely require a molecular diagnostic tool.

Many of the references below use what is here recognized as *C. sonorensis* as *C. variipennis*. It appears that *C. sonorensis* is the only BTV and EHDV vector within the *C. variipennis* complex. Serological surveys of populations of *C. occidentalis* and *C. variipennis* from various regions of the USA have shown very low infection rates (1-2%) [187]. This is

likely to be true for *C. albertensis* as well but remains to be tested. The inability to accurately separate *C. albertensis*, and *C. australis*, from *C. sonorensis* will assuredly affect the epidemiological data from regions where these species are sympatric. Consequently, this has also led to artificially expanding the distribution of *C. sonorensis* into Ontario and has likely caused an overestimated this species importance as a vector in the northeastern USA and Canada. Not only this, but it may have also delayed efforts to investigate the true primary vectors in these areas. Unfortunately, much of the early work focusing on BTV and EHDV do not make the distinction between the species of the *C. variipennis* complex and so disentangling this information can be difficult. For example, Smith and Stallknecht (1996) sampled *Culicoides* from deer farms in Georgia, Mississippi, and North Carolina during an outbreak of HD. They collected what they refer to as *C. variipennis*; however, so few individuals were collected that they concluded this species is unlikely to play a role in transmission. Jones *et al*. (1977) also collected what they referred to as *C. variipennis* from Kentucky but report that it was the most abundant species collected and was observed regularly attacking deer. Additionally, EHDV was isolated from this population and was also found to be susceptible to BTV. Clearly defining the species ranges of the members of the *C. variipennis* complex should be given high priority in order to determine where *C. sonorensis* is responsible for the transmission of BTV and EHDV.

Tabachnick (1996) cites unpublished data that shows specimens morphologically identified as *C. australis* were electrophoretically identified as *C. sonorensis*. Holbrook *et al*. (2000) reports that electrophoretic identification was done on specimens from Saltsville, Virginia (the type locality for *C. australis*) and reports that these were primarily *C. variipennis* with a few *C. sonorensis*. Holbrook *et al.* (2000) excluded this population from further analysis, potentially due to a low number of individuals; however, there seems to be little genetic evidence for the

exclusion of *C. australis* from species level status. Isozyme markers were unable to differentiate *C. sonorensis* from *C. albertensis* and so this may also be true for *C. australis*. Chapter II did not sample populations of *C. australis* when determining *C. albertensis* to be a valid species and thus we feel this warrants further study. Additionally, the primary habitat of *C. australis* is reported as salt brine pools which neither *C. sonorensis* or *C. variipennis* seem to prefer. The immature stages of *C. sonorensis* have been shown to be intolerant of high saline levels under laboratory conditions [370]. Vaughn and Turner (1987a) collected females from Saltsville, Virginia and brought them back to the lab to induce oviposition. They note that the immatures took longer to develop and had a reduced survival rate as compared to *C. variipennis* collected in New York [371]. As Vaughn and Turner (1987a) followed the same larval rearing protocol of Mullens (1983) perhaps these individuals were in fact *C. australis* and this low survival rate was an artifact of rearing these immatures in deionized water rather than salty water.

[8, 12, 17, 181, 372-375]

***Culicoides stigma* (Meigen)**

Ceratopogon stigma Meigen, 1818:73. Type locality: Europe, no holotype designated.
Ceratolophus stigma: Kieffer, 1906:61.
Culicoides kiefferi Goetghebuer, 1910:96. Type locality: Brussels, Belgium. Holotype ♀ (location unknown), 27 May 1910.
Culicoides cordiformitarsis Carter, 1916:134. Type locality: Kairo, Egypt. Holotype ♀ (location unknown), December, 1909.
Culicoides unimaculatus Goetghebuer, 1920:57. (unnecessary new name for *C. kiefferi* Goetghebuer, 1910:96).
Culicoides stigma: Goetghebuer 1921:49 (as synonym of *C. kiefferi* Goetghebuer, 1910); Goetghebuer 1922:59 (as synonym of *C. unimaculatus* Goetghebuer, 1920).
Culicoides (*Monoculicoides*) *stigma*: Khalaf 1954:40.
Culicoides stigmoides Callot, Kremer and Deduit, 1962:166. Type locality: Ardennes, France.

DIAGNOSIS: Male and female: only species of *C.* (*Monoculicoides*) with a black scutum. In addition, male: only species of *C.* (*Monoculicoides*) with a distinct posterior projection medial to

base of apicolateral process and with gonostylus gradually tapering distally; female: only species of *C*. (*Monoculicoides*) with a black scutum and with a finger-like extension on the spermatheca.

DESCRIPTION: *Male*, **Head**: eyes separated by 2 ommatidia. **Thorax:** scutum black with lighter hour-glass pattern on posteromedial area; wings without pattern of pigmentation. **Abdomen:** abdominal segment 1 with 5-7 pleural setae; epandrium with nearly parallel lateral margins, nearly parallel lateral margins, with distinct posterior projection medial to base of apicolateral process, equal to or longer than apicolateral process; apicolateral process with wide at base, tapering to apex; fused parameres with thick medially, short, stout base, apices narrowly separated; aedeagus triangular, without spicules; gonostylus gradually tapering distally. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** abdominal segment 1 with 6-9; spermatheca spherical, with finger-like extension, with spermathecal ring present.

DISTRIBUTION: *Culicoides stigma* is Holarctic, occurring in most of Europe (absent from the Balkans) south to northern Africa, east throughout much of the "forested zone" of Russia and far north China (Heilongjiang province). Also known from central Alberta, Canada. [26, 58, 77, 81-85, 87, 136, 196, 206, 248, 376-392]

Bionomics

Adult Habitat and Seasonality

In most of its distribution, *C. stigma* is active from May – September [58, 248, 381], though in its subarctic distribution, this species becomes active in late June and adult activity lasts only two

or three months [87, 393]. Although this species is widely distributed it is rare in most places [77, 390, 394]. Large populations of *C. stigma* have been reported near Perm, Russia [58].

Immature Habitat

Culicoides stigma has been reared from mud at the margins of ponds [248] and riverbanks [70], and the largest numbers can be found in silted waters [58]. In the UK, this species is found in association with livestock and has been collected from bare mud surrounding contaminated water, muddy hoof prints, and other temporary habitats [15]. It is also found living sympatrically with *C. nubeculosus* and *C. parroti* [135, 164]. In Belgium, *C. stigma* was also found in similar farmyard habitats, but was additionally collected in the wet soil between silage reserves [142, 395]. Edwards (1939) reported that this species was reared from green algae at the edge of a pond, though this association is not known elsewhere. The majority of *C. stigma* larvae have been collected from fresh water; however, a few specimens in Kettle and Lawson (1952) were also collected from a salt marsh.

Feeding

Culicoides stigma is commonly reported as feeding on both cattle and equids [380, 396, 397], primarily on the belly [197, 398]. This species has been collected from light traps on sheep farms [206, 399]; however, it has not been collected directly from sheep. While investigating the biting rates of *Culicoides* in Denmark, Elbers and Meiswinkel (2014, 2015) collected *C. stigma* from cattle and horses but noted that it was absent from sheep. This species has been reported to occasionally bite man at sunset [248, 381].

Mating behavior

Unknown

Development

In the Russian Republic of Karelia, Remm (1956) reported that *C. stigma* has two generations per year and Glukhova (1989) considered this species to be polycyclic. In areas with a longer warm season, this species is likely to have more generations.

Vector status

While *C. stigma* is associated with many livestock, there is no evidence that it plays a role in any viral pathogen transmission. In screening for Schmallenberg virus in France, one specimen of *C. stigma* was tested and found to be negative. The larvae of this species are however a known host for numerous aquatic parasites including horsehair worms (Gordiacea) [15], *Onchocerca* sp. [400], and flukes [401]. Because of this association, *C. stigma* has been implicated in the transmission of these parasites to vertebrate hosts. While not a pathogen, a *Rickettsia* endosymbiont was isolated from adults *C. stigma* [402].

Molecular data

Gene sequences of the COI [386], CYTB, and ITS1 [164] are available for this species. Augot *et al*. (2013) showed that these genes were successful in separating *C. stigma* from *C. parroti*.

Taxonomic Discussion

Culicoides stigma was among the first *Culicoides* ever described, though originally, it was placed in the genus *Ceratopogon* [403]. It was later incorrectly moved to *Ceratolophus* (a synonym of *Serromyia*) [404], most likely because of the lack of wing and scutellar patterning. In 1910 to 1916 two species which became synonyms of *C. stigma* were described from specimens collected in Belgium (*C. kiefferi*) and Egypt (*C. cordiformitarsis*). Goetghebuer (1920) designated *C. unimaculatus* as an unnecessary name for *C. kiefferi*. Goetghebuer (1921) reported both *C. kiefferi* and *C. unimaculatus* to be synonyms of *C. stigma* stating no discernable differences between them. Khalaf (1954) considered this species to be part of the *nubeculosus*

group within the newly formed subgenus *C*. (*Monoculicoides*). A final synonym was described in 1962 from specimens collected in France. Callot *et al*. (1962) stated that they described *C. stigmoides* because the drawing of the male *C. stigma* in Edwards (1939) did not depict spicules on the ventral surface of segment 9. There were no differences found between female specimens. As *C. stigma* males do have spicules on the ventral surface of segment 9, this was simply an omission in Edwards (1939) and *C. stigmoides* is clearly a synonym of *C. stigma*. Further adult descriptions can be found in (Glukhova 2005). We have examined a specimen from Canada and agree that it is indeed *C. stigma*. The Nearctic distribution of this species is likely localized to north west Canada and this population was perhaps contiguous with the rest of its distribution when there was a land bridge connecting Russia and Alaska.

Kettle and Lawson (1952) were the first to describe the larvae and pupae of this species. When compared to *C. nubeculosus* and *C. riethi*, the larvae of *C. stigma* were markedly smaller and with the labium not strongly sclerotized. Similarly, they noted the pupa of *C. stigma* was the smallest within the group. Other larval descriptions exist in Glukhoava (1968a, 1968b, 1977, and 1979), Gutsevish and Glukhova (1970), Spătaru (1971), Knoz (1980), and Chaker (1983). The character used in Glukhova (1968a, 1968b) to separate the larvae of *C. stigma* from other members of *C*. (*Monoculicoides*) is the "sensilla on the labium lying on distance less than the base of the sensilla". Chaker (1983) notes that the larvae of *C. stigma* can be distinguished based on a thick, strongly sclerotized post-occipital collar and the presence of a long longitudinal slit on the ventrolateral scleritis. Other pupal descriptions can be found in Dzhafarov (1964), Spătaru (1971), and Shults and Borkent (2018). Shults and Borkent (2018) note that the dorsal apotome of *C. stigma* is covered in small, uniform spines compared to those in *C. sonorensis* and *C. riethi*.

Kettle and Lawson (1952) draw the dorsal apotome of *C. parroti* and this looks very similar to *C. stigma*.

[392, 405]

Culicoides taonanensis Ren, Wang, & Liu

Culicoides (*Monoculicoides*) *taonanensis Ren, Wang*, *& Liu,* 2006:388. Type locality: Taonan County, Jinlin, China. Holotype ♀ slide (Center for Disease Control and Protection, Shenyang Command), 15 July 2005; Allotype ♂ slide (same place), same collection information; Paratypes 5 ♀ (same place), same collection information.

DIAGNOSIS: Male: only species of *C*. (*Monoculicoides*) with a reduced apicolateral process; female: morphologically similar to most species of. (*Monoculicoides*) with spherical spermatheca.

DESCRIPTION: *Male*, **Head**: eyes an unknown distance apart. **Thorax:** scutum unknown; wings with faint pattern of pigmentation. **Abdomen:** epandrium with nearly parallel lateral margins, caudal margin with V-shaped medial notch; apicolateral process with wide at base, tapering to apex, short; fused parameres with long, slender base, apices narrowly separated; aedeagus triangular, without spicules; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**. Abdomen:** spermatheca ovoid, with spermathecal ring absent.

DISTRIBUTION: *Culicoides taonanensis* is known only from northeast China from eastern Inner Mongolia, Jilin, and Liaoning [406, 407].

Bionomics

Adult Habitat and Seasonality

The ecoregion of China where *C. taonanensis* occurs is a temperate broadleaf and mixed forest [16].

Feeding

With 12 fine teeth on the mandibles [407], *C. taonanensis* likely feeds on vertebrates.

Little else is known about this species.

Taxonomic Discussion

In the original description of *C. taonanensis*, Ren *et al*. (2006) recognized this species as a member of *C*. (*Monoculicoides*) and we would agree with this assessment based on the presence of a dark spot below the arculus.

***Culicoides variipennis* (Coquillett)**

Ceratopogon variipennis Coquillett, 1902:602, three syntypes ♀; Richmond, Virginia, USA; Westville, New Jersey, USA; Mexico City, Mexico, Catalogue # 5465, (USNM). Lectotype designated by Wirth & Jones, 1957:12 as the syntype from Virginia.
Culicoides variipennis: Kieffer, 1906:55.
Culicoides (*Monoculicoides*) *variipennis*: Khalaf, 1954:40.
Culicoides variipennis variipennis: Wirth & Jones, 1957:12 as a subspecies of *C. variipennis*.
Culicoides variipennis: Downes 1978:63; Holbrook *et al*., 2000:68.

DIAGNOSIS: Male and female: only species of *C*. (*Monoculicoides*) with prominent black spots and brown vittae on scutum, and with sensilla coeloconica present on only on flagellomeres 1, 6-8. In addition, male: only species of *C*. (*Monoculicoides*) caudal margin of S9 straight, and with aedeagus bare or with spicules only at apex; female: only species of *C*. (*Monoculicoides*) with third palpal segment narrow.

DESCRIPTION: *Male*, **Head**: eyes separated by 2-3 ommatidia. **Thorax:** scutum grey with black spots; wings with well-defined pattern of pigmentation. **Abdomen:** abdominal segment 1

with 20-29 pleural setae; epandrium with nearly parallel lateral margins, caudal margin straight; apicolateral process with narrow at base, tapering to apex; fused parameres with long, stout base, apices narrowly separated; aedeagus triangular, without spicules or with spicules only at apex; gonostylus tapering gradually for basal half, with apical portion slender. *Female*, as is male but with these differences, **Head**: eyes broadly separated by a distance equal to 2-3 ommatidia on the dorsal portion of the head, sensilla coeloconica on flagellomeres 1, 6-8; palpus with third segment narrow with small pit; mandibular teeth well developed. **Thorax:** no difference**.** **Abdomen:** abdominal segment 1 with 6-10; spermatheca ovoid, long, C-shaped, with spermathecal ring absent.

DISTRIBUTION: *Culicoides variipennis* occurs from in three disjunct regions of the Nearctic Region, as follows: from southern British Columbia, Canada, south to Washington and Montana, USA; Wisconsin, USA east to southern Ontario and Quebec, Canada, south to east Texas and Florida, USA; in the vicinity of Mexico City, Mexico [17, 19, 23, 167, 188, 408-416].

Bionomics

Adult Habitat and Seasonality

This species was collected from May to October in most of its distribution, with peak emergence during the summer months [17, 23, 409, 417-419]. Interestingly, Mullens and Ruts (1984) collected more females throughout the night and at dawn than were collected at dusk. Other members of the *C. variipennis* complex are most abundant at dusk.

Immature Habitat

Culicoides variipennis larvae live in the margins of freshwater ponds and streams and can reach high larval densities in water contaminated with organic material, often associated with farmyards [167, 291, 417, 420]. Schmidtmann *et al*. (1983) found this species most often in

habitats containing approximately 12-16% organic material. However, any habitat approaching 50% organic material was nearly devoid of *C. variipennis* larvae. *Culicoides variipennis* and *C. sonorensis* can be collected in the same contaminated habitat in some areas of the southeastern USA as well as Kansas, Missouri, Oklahoma, and Arkansas [19, 23] Shults *et al.* (2021). Schmidtmann *et al*. (2000) measured the soil chemistry of the larval habitats of *C. variipennis* across the entirety of its geographic distribution. In general, the concentrations of the minerals tested (borons, phosphates, etc.) were relatively low in habitats where *C. variipennis* was collected. This was also confirmed in a follow-up study [176]. There were slight differences found in the habitat preference between *C. variipennis* and *C. sonorensis*; however, this is likely due to the inclusion of *C. australis* individuals identified as *C. sonorensis* in these studies.

Feeding

Culicoides variipennis is associated with farmyards and will readily fed on a variety livestock including deer, swine, cattle, and horses, and are occasionally known to attack humans [23, 169, 328, 417, 421, 422]. Mullens and Rutz (1984) classified this species as an opportunistic feeder as the females only attacked researchers as they approached an area containing cattle. These attacks also only took place during the day, an observation also made from a population in Tennessee in Pickard and Snow (1955) and Snow *et al*. (1957). In the lab, *C. variipennis* has been fed artificially on animal blood as well as directly on humans [167, 291].

Mating behavior

In Downes (1978), eggs were obtained from numerous field-collected female *C. variipennis* from Ontario, Canada. These eggs were successfully reared to adults; however, no mating was attempted in captivity [167]. This failure to establish an F1 generation was presumed to be because *C. variipennis* is an obligate swarmer, and in fact, swarms are often observed at farm

sites [167, 352]. Jones and Schmidtmann (1980) had better luck at propagating this species in the lab though it seems only a single population was able to produce an F2 generation. The success of this colony line was determined to be due to the fact that some of the field-collected individuals possessed the ability to mate in confined spaces. They theorized that this trait was rare in most populations and by starting the colony with adults rather than pupae (the collection methods used in previous colonization attempts), they increased the chances of capturing individuals with this ability [352]. This seems plausible as the genetic diversity in a single larval habitat is potentially limited, but this is likely not the case for adults collected from light traps.

Development

Childres and Wing (1968) estimated that *C. variipennis* completed between 3-5 generations in central Missouri. Mullens and Rutz (1983) determined that the average number of degree days required for *C. variipennis* to complete development was 285 [371]. Depending on the weather conditions, this would equate to approximately 10 – 16 days during the summer months. Mullens and Rutz (1983) used individuals collected in New York to obtain this data, but similar developmental times were also obtained from a population collected in Virginia [423]. The main difference found between these two studies was the larval survival rate. Mullens and Rutz (1983) reported a survival rate of 60-70%, while Vaughan and Turner (1987) reported survival rates of 10-18%. Both of these studies reared eggs from field-collected females and raised the larvae on substrate from the natural habitat. *Culicoides australis* is also known from that area and perhaps the population that Vaughan and Turner (1987) used was not purely *C. variipennis*. As these two species live in different larval habitats, collecting both as adults and then rearing the subsequent immatures on a single substrate would undoubtedly lead to higher mortality in one than the other. Female *C. variipennis* have been collected in the field having completed at least four

gonotrophic cycles and were estimated to be 19 days old [291]. Females started host seeking 1-2 days after emergence and follicle growth started even before the first sugar or bloodmeal. No autogeny was observed in this species [291]. In May 1985, 51% of the larvae of *Culicoides variipennis* collected from mud in a horse pen at Auburn, Alabama, were found to be infected with a species of *Heleidomermis* tentatively identified as *H. vivipara* [424].

Vector status

This species is considered to be a poor vector as populations known to predominately be *C. variipennis* have yielded low (> 1.0%) seroprevalence rates [425, 426].

Molecular data

Both isozyme and SNP data have been used as evidence of the species status of *C. variipennis* [19, 427]. Mitochondrial sequences of this species are available [6, 181, 369]; however, these genes have been unsuccessful in separating *C. variipennis* from *C. sonorensis.* Chapter II proposed that this could be due to hybridization and introgression. The nuclear genes CAD, TPI, and E1alpha are also available for this species [188, 369].

Taxonomic Discussion

Female adult *C. variipennis* are one of the most easily recognizable species of the *C. variipennis* complex as they have a distinctly narrow third palpal segment and a dark pattern on the wing. The original description of *C. variipennis* was based on specimens from three separate localities but it is in agreement with typical character states for individuals in the *C. variipennis* complex. It was originally described as a species of *Ceratopogon* [428], but Keiffer (1906) later moved it into *Culicoides*. Khalaf (1954) noted the similarity of this species to the European species *C. nubeculosus* and placed it in the *nubeculosus* group within the subgenus *C*. (*Monoculicoides*). Wirth and Jones (1957) noticed both morphological and ecological differences between

populations of *C. variipennis* throughout North America and this led them to describing five subspecies of *C. variipennis*. Wirth and Jones (1957) also designated the Virginia syntype from Coquillett (1902) as the lectotype for *C. variipennis* and this specimen represented the subspecies *C. variipennis variipennis*. Both Jorgensen (1969) (in part) and Downes (1978) agreed that there was enough morphological evidence to warrant species status for *C. variipennis* and this was later supported genetically by Holbrook *et al*. (2000).

It is difficult to know what species some workers were referring to in descriptions and keys prior to Wirth and Jones (1957), other than inferences from distributions, which vary among at least some of the species in the *C. variipennis* complex (see discussion under "*C. variipennis* complex"). The syntype females collected in New Jersey and Virginia are likely to be *C. variipennis*; however, the specimen from near Mexico City, Mexico could equally be *C. sonorensis* or *C. occidentalis, which also occur there* [412]. Additionally, many of the redescriptions of the adults of *C. variipennis* were done using individuals from multiple locations and thus are designated as *C. variipennis* (in part) including Malloch (1915), Hoffman (1925), and Foote and Pratt (1954). The descriptions of *C. variipennis* in Root and Hoffman (1937) and Wirth (1952) refer to *C. sonorensis* and *C. occidentalis* respectively. The adult descriptions of *C. variipennis* (as *C. variipennis variipennis* until Holbrook *et al*. (2000)) that appear to be wholly of this species are Jones (1955), Wirth and Jones (1957), Jamnback (1965), Battle and Turner (1971), Blanton and Wirth (1979), and Holbrook (2000). It should be noted that the description of the male in Jamnback (1965) was adapted from Wirth and Jones (1957) as no males were collected in his study.

The pupa of this species was first briefly described by Malloch (1915); however, it appears that the specimens used were reared from a salt spring and would more likely be *C.*

australis. Therefore, the first pupal description of confidently identified *C. variipennis* was by Thomsen (1937) from specimens collected in New York. Additional descriptions of the pupae of *C. variipennis* can be found in Fox (1942), Jones (1955), Blanton and Wirth (1979), and Shults and Borkent (2018). One of the specimens identified as *C. variipennis* in Shults and Borkent (2018) would appear to be *C. australis* based on being collected from a salt spring. The pupal descriptions of *C. variipennis* in Wirth (1952) are of *C. sonorensis* and *C. occidentalis* as he collected specimens from wastewater ponds and saline pools. Notably, Jamnback (1965) did not collect the pupa of *C. variipennis* during his study and instead based his description on a specimen of *C. australis*.

Jones (1955) provided the first description of the fourth instar larva of *C. variipennis* collected from waste water ponds in Wisconsin. Further descriptions of the larva are in Blanton and Wirth (1979), Murphree and Mullen (1991), and Hribar and Mullens (1991). Again, as Jamnback (1965) did not collect immatures of *C. variipennis* during his study, he instead used a specimen of *C. australis* for his larval description. Using a principle component analysis, Hribar and Mullens (1991) found significant divergence between the larvae of *C. variipennis* and *C. occidentalis*; however, no differences were apparent between *C. variipennis* and *C. sonorensis* in Abubekerov and Mullens (2018).

From a zoogeographical stand point, *C. variipennis* has an interesting distribution as there seems to be three disjunct populations; one in the Pacific Northwest, one generally in eastern USA, and another in central Mexico. The Pacific Northwest population is restricted to southern British Columbia, eastern Washington and western Montana. We have examined specimens from southwest Wyoming labelled as *C. variipennis* and have determined these to be *C. albertensis.* It is possible that the Pacific Northwest *C. variipennis* are contiguous with the eastern distribution

by way of northern Canada, but this seems unlikely considering it is otherwise restricted to near the southern border of Canada [22]. This species appears to at least be absent from the Canadian prairies. The population in Mexico is almost certainly a relic of an ancestral, more broadly distributed population and appears isolated. We have examined one female specimen collected near Mexico City and it appears to be a typical *C. variipennis*. Two other specimens from near Mexico City were also identified as *C. variipennis* by Huerta (2012). Vargas (1945) reported *C. variipennis* from the state of Oaxaca, Mexico; however, we are unsure as what species he was actually referring to and no specimens appear to be available for study. This record represents the furthest southern distribution of any North American *C.* (*Monoculicoides*) species and the only one in the Neotropical Region. Macfie (1948) included *C. variipennis* in a key to species of Chiapas, Mexico, based in part on species reported from neighboring regions and indicating that *C. variipennis* was only included because of the Vargas (1945) report. No *C. variipennis* were actually collected in Chiapas and we highly doubt that that this species would occur in the Neotropical Region. Similarly, in the 1940's, there were reports of *C. variipennis* from Venezuela, but these were later clarified as misidentifications by Otriz and Mirsa (1952). [11, 12, 19, 177, 418, 429-437].

Discussion

The Culicoides variipennis complex

The species of the *C. variipennis* complex were first described as a single species, *Ceratopogon variipennis* by Coquillett (1901), and was later moved to *Culicoides* by Kieffer (1906). Originally, this species was recognized as being present in New Jersey, Virginia, and Mexico, but soon was recorded as present in most of North America [404, 410, 433, 436-442].

Mayer (1934) placed *C. variipennis* within the nubeculosus group [272] based on the pupal description of Malloch (1915). Khalaf (1954) included the nubeculosus group within his in his newly described subgenus *C*. (*Monoculicoides*), thus placing this species into its current subgenera. Wirth and Jones (1957) noted morphological variation in the adults of *C. variipennis* originating from differences in geography and larval habitat, and thus formally described five subspecies of *C. variipennis*: *C. variipennis variipennis*, *C. australis, C. v. sonorensis*, *C. v. albertensis*, and *C. v. occidentalis*. The morphological characters noted as important to differentiate these subspecies were: in the females, the ratio of the length to width of the third palpal segment, length of the wing, which flagellomeres contained sensilla coeloconica, the number of mandibular teeth, and to a lesser extent, the shape of the spermatheca, pigmentation pattern on the scutum, the wing pattern, and in the males, the number of spicules on the aedeagus. However, there was a substantial overlap in the character states and morphometric data between all five subspecies. Table 2 of Wirth and Jones (1957) lists the mean measurement of several diagnostic characters and the 95 percent limits rather than the raw range of these values. This may seem as though there are clear delineations between species for these characters, though in reality, this is not the case. For example, the wing length of *C. sonorensis* is listed as 1.16-1.36 mm; however, figure 6 of Wirth and Jones (1957) clearly shows a far greater amount of variation within this species. Specimens of *C. sonorensis* from Utah and Texas had wing lengths greater than 1.50 mm. Additionally, the differentiation of some subspecies was based solely on size and coloration which are known to vary biogeographically and seasonally [443-447]. Studies have also shown that adult size in species of *Culicoides* is directly linked to environmental factors, the nutritional composition of the larval habitat, and even larval densities [317, 448, 449]. Wirth and Jones (1957) also noted introgression in the number of spicules on the

aedeagus and width of the 3rd palpal segment in areas of sympatry between several subspecies. They mention that this could potentially be due to hybridization or ecological character displacement [450-453]. As pointed out by Atchley (1967), Downes (1978), and Wirth and Morris (1985), the complexity and variation in the geographic distribution of the characters listed in Wirth and Jones (1957) may be insufficient to accurately and consistently identify the subspecies as recognized by these authors within the *C. variipennis* complex.

Due to this ambiguity, a variety of phylogenetic groupings and taxonomic arrangements have been proposed for the species in the *C. variipennis* complex [22-24, 169, 172, 449, 454-456]. An in-depth summary of these was reported by Wirth and Morris (1985). Most works theorized that there were two main lineages; *C. variipennis* in the eastern U.S.A. with little morphological variation and a western species with highly variable characters. Atchley (1967) synonymized *C. australis* with *C. v. sonorensis* as he was unable to distinguish these two subspecies in New Mexico. He suggested that the morphological variation observed in Wirth and Jones (1957) may constitute ecotypes rather than true species. In studying specimens of the *C. variipennis* complex in British Columbia, both Jorgenson (1969) and Downes (1978) found that *C. variipennis* and *C. occidentalis* could be collected in sympatry with no introgression of characters, and therefore represented valid species. Wirth and Morris (1985) note that some of the specimens Jorgensen (1969) described could have partially or wholly included *C. sonorensis* rather than *C. occidentalis*. Downes (1978) formally recognized *C. variipennis* and *C. occidentalis* as species and designated the remaining three taxa as nominotypical forms of *C. occidentalis*. Wirth and Morris (1985) mentioned that they preferred the use of the species name *C. sonorensis* over *C. occidentalis*, but never formally changed this designation. Holbrook *et al*.(2000) used genetic markers (discussed in detail later), morphology, and larval habitat to elevate *C. sonorensis* to full

species status. As they were only able to find genetic and morphological evidence for *C. occidentalis*, *C. sonorensis*, and *C. variipennis*, the two remaining subspecies, *C. v. albertensis* and *C. australis*, were designated as synonyms of *C. sonorensis*. Holbrook *et al*. (2000) also provide a key to these three species (though figure 3A and 3B should be interchanged) but were unable to morphologically separate female *C. occidentalis* from *C. sonorensis* or male *C. variipennis* and *C. occidentalis*. Finally, Shults *et al*. (2021) (discussed in detail later) used double digest restriction-site associated sequencing (ddRAD-seq) to generate a dataset of single nucleotide polymorphisms (SNP) to examine individuals of the *C. variipennis* complex from 17 populations around the U.S.A. and Canada. This study found five distinct and divergent genetic clusters inferred from the SNP loci, and these clusters were assigned to *C. albertensis, C. occidentalis*, *C. sonorensis*, *C. variipennis*, and the previously undescribed species from San Diego, California; *C. mullensi*. This study provided molecular-based evidence for the species status of *C. albertensis* and *C. mullensi*, which here we have shown morphological evidence for as well.

The larvae of the different taxa within the *C. variipennis* are associated with different habitat types [17, 23, 172, 183, 304, 457]. *Culicoides sonorensis* and *C. variipennis* are found in sewage effluents and ponds, streams, intermitted flows, or bogs contaminated with manure [17, 458]. *Culicoides albertensis* is found in alkaline pools and *C. australis* and *C. occidentalis* breed in highly saline ponds and lakes. Atchley (1967) was unable to support these findings as he reared pupae from a highly saline lake in New Mexico and found that these specimens were *C. sonorensis* rather than *C. occidentalis* or *C. australis*. His identification was based on the presence of only a few spicules on the distal end of the aedeagus, but again, table 2 of Wirth (1957) is misleading in listing *C. australis* as having a bare aedeagus. Again in Wirth and Jones

(1957), figure 4 clearly shows this character to be quite variable within this subspecies. Additionally, spicules at the distal end of the aedeagus is also the character state for *C. occidentalis* and *C. sonorensis* hybrids. Childers and Wingo (1968) collected both *C. sonorensis* and *C. variipennis* in sewage lagoons in Missouri and also reared *C. australis* from a salt spring. *Culicoides occidentalis* has been collected from many highly saline and alkaline habitats in the western U.S.A. [19, 167, 172, 183]. With the reclassification of the *C. variipennis* complex by Holbrook *et al*. (2000), Schmidtmann *et al*. (2000) set out to characterize the soil chemistry of the larval habitat for each species. They found that habitats high in phosphate, organic material, and nitrate, as found in livestock waste, supported *C. sonorensis* and *C. variipennis*. This association has been well documented [304, 459, 460] and Mullens and Rodrigues (1988) showed that as fecal pollution in waste water tanks increased, so did the larval density of *C. sonorensis*. There is however an upper limit at which the percentage of organic material in the environment makes the habitat inhospitable to the larvae of this species, possibly due to reduced oxygen content [458]. Schmidtmann *et al*. (2000) also found salt-forming ions and other indicators of salinity were elevated in habitats supporting *C. occidentalis*, *C. sonorensis*, and *C. variipennis*, as compared to fresh water, but habitats supporting *C. occidentalis* had a unique soil composition. Specifically, boron and chloride levels were much higher while phosphate levels were low. Occasionally, *C. sonorensis* and *C. occidentalis* can be collected from the same larval habitats [19, 177]. Studies have shown the eggs of *C. sonorensis* are somewhat resistant to desiccation and the larvae can survive at certain levels of salinity [370, 461]. This plasticity may allow them to reproduce in habitats outside of their preferred sites. However, there may be an upper limit to the amount of salinity this species can handle. Using *C. sonorensis* females obtained from a laboratory colony, Linley (1986) found that females laid fewer eggs in water

with higher salinities, and no eggs were laid when the salinity was 34.0 parts per thousand (ppt). There was no significant difference in hatch rate or survival of *C. sonorensis* eggs laid in 0.0, 9.9, and 19.0 ppt. This is unsurprising as waste-water troughs and sewage lagoons are often between 5-10 ppt [317]. Additionally, any *C. sonorensis* eggs laid in a low saline treatment and then transferred to this high saline treatment were unable to hatch. By comparison, *C. occidentalis* larvae have been collected from Mono Lake in California where the salinity is approximately 88.0 ppt [462]. If >30 ppt is really the upper limit for *C. sonorensis* this could represent a means of reproductive isolation via ecological isolation. If this is the case, it remains to be seen as to which species is living in the salt pools, salt flats, salt brines, and salt marshes of the Mississippi Valley and Gulf Coast plains of the USA. These are the specimens described as *C. australis* in Wirth (1957). Either natural populations of *C. sonorensis* have adapted to higher salt tolerance in these localities but not elsewhere (ecotypes), or *C. australis* is in fact be a valid species.

The ability to find distinct morphological forms in sympatric larval habitats provides evidence of sympatric species, though some form of reproductive isolation must exist to maintain species boundaries. Veltan and Mullens (1997) showed a lack of post-zygotic reproductive isolation between *C. sonorensis* and *C. occidentalis* alluding to the presence of pre-zygotic isolation. One possible mechanism for this is the swarming behavior exhibited by each species. The males of both *C. variipennis* and *C. occidentalis* have been observed swarming near their respective larval habitats [167, 183], whereas *C. sonorensis* males are attracted to CO^2 and usually breed at or near a host [182, 189, 315, 463]. The differences in swarming behavior alone would not stop matings between *C. variipennis* and *C. sonorensis* as they share both a host and larval habitat, but would be sufficient to limit mating between *C. occidentalis* from *C.*

sonorensis. In *C. nubeculosus* and *C. melleus*, previously mated females were less receptive to additional matings, thus *C. occidentalis* females mated at the larval habitat could be less likely to mate with *C. sonorensis* males at the host [152, 464]. However, this type of reproductive isolation may not be impermeable as virgin females can become less selective with age. Additionally, the salinity of the larval habitat where *C. occidentalis* females deposit their eggs may also be too high for the hybrids to survive. Though even if the hybrids were to survive, they would have an increased chance of mating with *C. occidentalis* males, leading to directional backcrossing. The hybrid individuals collected in northern California in chapter II were collected from a saline pool and both had *C. occidentalis* as their maternal lineage, supporting the scenario described above. Zimmermann *et al*. (1982) gives a detailed description of the swarming behavior of the *C. variipennis* complex around brine pools near Saltville, Virginia. Males formed swarms at sunset, usually over something with a dark contrast to the surrounding environment such as clumps of grass, dark soil, or artificial markers. As females entered the swarm, males coupled with them and the pair fell to the ground to finish mating. Almost exclusively, these females were virgin, likely indicating that a single mating is sufficient to fertilize multiple batches of eggs, as has been confirmed to be the case in *C. sonorensis* [465, 466].

The development of molecular diagnostic tools allowed for the differentiation of independent species as well as the estimation of their genetic divergence. Isozymes were the first molecular markers used to examine populations of the *C. variipennis* complex [368]. Approximately 30 different enzyme systems were tested and of those, 17 were found to be polymorphic allowing for the analysis of 21 loci (some enzymes contained more than one locus). Tabachnick (1990) used laboratory colonies of *C. sonorensis* as a means of testing these markers against various field populations. He found the use of allele frequencies alone could differentiate *C. sonorensis*

from *C. variipennis*. A follow-up study was conducted using the same markers but testing a wider range of wild populations and found that there were at least three independent populations within the species complex, *C. occidentalis*, *C. sonorensis*, and *C. variipennis* [25]. Based on the overall genetic similarity, it was found that *C. occidentalis* was the sister taxon to the other two species. Three studies then used the most polymorphic isoenzymes (7-11 loci) from Tabachnick (1992) to analyze populations of the *C. variipennis* complex from California [175], the New England area [427], and Virginia [20]. Each found a high degree of differentiation between species with little variation within populations of the same species. The molecular markers used by Tabachnick (1992) were chosen specifically for the high degree of differentiation between *C. occidentalis*, *C. sonorensis*, and *C. variipennis*. This artificially increased the genetic differentiation between these species (table 3.1) and potentially nullified the ability to identify *C. albertensis* and *C. australis*. A final allozyme study was conducted using seven isozymes (7 loci) to examine individuals collected from larval habitats throughout most of the southern USA. [19]. The results from the genetic and morphological analyses showed strong evidence of three independent species, *C. occidentalis*, *C. sonorensis*, and *C. variipennis*. Neither molecular markers nor morphology showed evidence of introgression, hybridization, or the presence of further species. This was consistent with the previous population genetic studies; however, isozymes are not the best tools to identify certain levels of genetic variation [467]. Additionally, without "species-specific" alleles (which none of the studies mentioned above found), hybrids cannot be accurately detected. The lack of evidence of hybridization as well as the synonymizing of *C. albertensis* and *C. australis* with *C. sonorensis* was based primarily on morphology. Holbrook *et al*. (2000) mentions in the results that specimens identified as *C. australis* and *C. albertensis* were tested and were genetically similar to either *C. sonorensis* or *C. variipennis*, but

these specimens were not included in the statistical or phylogenetic analysis. Potentially, these markers were not sufficiently polymorphic to be able to differentiate the species.

Table 3.1. A summary of the Nei's genetic distance (D-distance) reported in population genetic studies examining the *C. variipennis* complex. The bold values indicate the mean genetic distance across all species-level pairwise comparisons. (o = *C. occidentalis*, s = *C. sonorensis*, and v = *C. variipennis*).

D-distance between species

Species Comparison	Tabachnick 1990	Tabachnick 1992*	Holbrook 1995	Holbrook 1996	Schmidtmann 1998	Holbrook 2000	Shults 2021
o + s	-	0.21	0.41	-	-	0.34	0.34
o + v	-	0.31	-	-	-	0.70	0.35
s + v	0.15	0.19	-	0.74	0.64	0.66	0.33
C. albertensis	-	-	-	-	-	-	**0.27**
C. mullensi	-	-	-	-	-	-	**0.28**

D-distance within species

Species	Tabachnick 1990	Tabachnick 1992*	Holbrook 1995	Holbrook 1996	Schmidtmann 1998	Holbrook 2000	Shults 2021
C. occidentalis	-	0.16	0.13	-	-	0.10	0.20
C. sonorensis	0.05	0.05	0.01	-	0.10	0.07	0.02
C. variipennis	-	0.03	-	0.05	0.08	0.14	0.02
C. albertensis	-	-	-	-	-	-	0.03

* – this paper used Nei's index of similarity (I) which has be converted to D distance using the formula, D = -ln(I) [468].

As barcoding has become common practice, Shults (2015), Hopken (2016), and Jewiss-Gaines *et al*. (2017) used sequencing data to try to differentiate between the species of the *C. variipennis* complex. The COI gene can identify *C. occidentalis*; however, there is little differentiation between *C. sonorensis*, and *C. variipennis*. The COII, CAD and TPI genes are unable to separate *C. sonorensis* and *C. variipennis* [188]. Jewiss-Gaines *et al*. (2017) reported the presence of *C. sonorensis* and *C. variipennis* in Ontario, Canada based on the morphology of the third palpal segment. They also reported high genetic similarity between these two species using the COI (100%), ITS1 (98%), and EF1a (99%) genes. Specimens identified as *C.*

sonorensis in this study were included in chapter II and were instead identified to be *C. albertensis*. I also sequenced the COI gene of specimens identified to species using SNP data and found that *C. albertensis*, *C. sonorensis,* and *C. variipennis* all shared mitochondrial haplotypes. These three species had less than 1.0% divergence of the COI gene, and in comparison, the differentiation among species (outside of this grouping) was around 3.0%; commonly associated with species-level divergence [469]. Additionally, the mitochondrial sequences of *C. occidentalis* from Borax Lake were highly divergent from *C. occidentalis* collected in British Columbia, Utah, and Nevada. Tabachnick (1992) showed this same divergence between the Borax Lake population and *C. occidentalis* from elsewhere in California. Whether a further cryptic species of *C. occidentalis* exists needs further investigation. This study showed that the inability to barcode the species of the *C. variipennis* complex was due to a biological phenomenon rather than the misidentification of specimens. The use of a single gene (or even the entire mitochondrial genome) may not allow for accurate identification of these species; however, with genomic data available, a more advanced molecular diagnostic tool could be developed.

With evidence from chapter II that the original subspecies designation constitutes true species, we have reexamined the morphology of the *C. variipennis* complex. Here we propose fresh character states for the recognition of species; however, these, like the characters in Wirth and Jones (1957) and Holbrook *et al*. (2000), may not be reliable in every population in North America. We first examined the variation within a single population of each species. The amount of variation within these populations, as well as the overlap between them, was informative as to what characters might be useful for species identification. The male characters examined were the antennal ratio, ratio of the 3rd palpal segment, proboscis length (in relation to wing length),

number of pleural setae of segment 1, number of spicules on the aedeagus, shape of the gonostylus, and shape of the paramere. For the females, we examine the same characters, other than the male genitalia, in addition to the number of flagellomeres with sensilla coeloconica, and spermatheca shape. *Culicoides sonorensis* males were easily identifiable with several characters that differentiated them from the other species of the *C. variipennis* complex. The ratio of 3rd palpal segment in *C. sonorensis* males was lower meaning that palps in this species are wider. This would make sense as *C. sonorensis* is the only species known to mate at the host and thus this adaptation in the males allows them to locate sources of CO_2. Additionally, *C. sonorensis* males had the fewest number of pleural setae on segment 1 (8-12) with *C. albertensis* having the second fewest with 15-17. The remaining two species, *C. occidentalis* and *C. variipennis* had 18-29 and 20-29 pleural setae respectively. As has been pointed out by both Wirth and Jones (1957) and Holbrook *et al*. (2000), we also found that *C. sonorensis* males always had numerous spicules on the aedeagus, whereas the aedeagus of *C. occidentalis* is always bare. *Culicoides albertensis* males have 3-8 spicules at the distal end of the aedeagus while *C. variipennis* males can have either spicules at the distal end as well as a bare aedeagus. Unfortunately, the presence of only a few spicules at the tip of the aedeagus is also a character state known from hybrids [183], thus its use as a diagnostic character outside of *C. sonorensis* and *C. occidentalis* may be limited. Finally, the shape of the gonostylus is diagnostic in *C. sonorensis* which gradually tapers to the distal end rather than tapering to the midpoint as seen in the other species. For males, the antennal ratio, the proboscis length/length of the wing, and the shape of the paramere were not found to be useful for species identification.

Similar to the males, the antennal ratio was uninformative for the purposes of species identification of the females. *Culicoides occidentalis* was found to have the widest 3rd palpal

segment (PR=1.86-2.33) follow closely by *C. sonorensis* (PR=2.25-2.50). Though there is some overlap between these species, in general, the palp of *C. occidentalis* is almost half as wide as it is long. As was shown in Wirth and Jones (1957) and Holbrook *et al*. (2000), *C. variipennis* females have a much narrower palp. The range of the palpal ratio of *C. albertensis* actually overlaps both *C. sonorensis* and *C. variipennis*. As this character is widely used for species delimitation, this likely explains some of the ambiguity and intermediate individuals reported in the literature. For *C. variipennis*, sensilla coeloconica were present on only four flagellomeres (1, 6-8). In *C. occidentalis* sensilla coeloconica were found on 1, 5-8 and in *C. sonorensis* on 1, 4-8. This character was highly variable for *C. albertensis*; however, the arrangement of sensilla coeloconica on flagellomeres 1, 3-8 was only observed in this species. In general, *C. mullensi* is larger than any other species in the *C. variipennis* complex; however, size can be misleading for diagnostic purposes as it can be affected by environmental factors. To compensate for this, we examined the length of the wings as a ratio with the length of the proboscis. The wing of *C. bajaensis* females was proportionally larger than the other species. Similar numbers of plural setae were observed between males and females with the exception of *C. variipennis*. Male *C. variipennis* have 20-29 setae while only 6-10 were observed in the females of the same population. The shape of the spermatheca was highly variable within each species and as such we were unable to interpret this character for diagnostic purposes. Morphological variation may be cryptic for the purposes of delimiting these species; however, the presence of ecological, behavioral, and genetic differences between the members of the *C. variipennis* complex provides evidence of speciation. For the purposes of vector surveillance though, the inability to accurately identify these species emphasizes the need for molecular diagnostic tools. [6, 17, 188, 248, 369, 370, 421, 428, 461, 470-474]

Conclusion

An enormous amount of physiological, epidemiological, and ecological data exists for just a few *C*. (*Monoculicoides*) species, and so there is a large discrepancy between what is known for these species as compared to the rest of the subgenus. By studying the entire group, biological and ecological patterns have emerged, and by utilizing the phylogeny, we can start to make informed hypotheses about this missing information. Throughout this study, it also became increasingly clear as to what patterns or connections mentioned in the literature were superficial. For example, almost all *C*. (*Monoculicoides*) feed on large mammals and this often puts them in close proximity to livestock. This association alone seems to be the only evidence for incriminating any *C*. (*Monoculicoides*), outside of *C. sonorensis*, as a potential vector species. In reality, there is almost no evidence to suggest that this group, again outside of *C. sonorensis*, plays any significant role in pathogen transmission. Which begs the question, what specifically makes *C. sonorensis* a highly competent vector? Especially as the *variipennis* complex appears to have undergone a relatively recent speciation event.

Our evidence suggests that the original observations made in Wirth and Jones (1957) about the *C. variipennis* complex were mostly correct. It would appear that the slight morphological and ecological traits used to designate subspecies actually delimit biological species. Wirth and Jones (1957) even hinted to the presence of *C. mullensi* but were not sure enough to warrant describing it. Our perception of how many species were in this complex were certainly informed by the SNP data presented in chapter II; however, there were very clear ecological clues to support *C. australis* and *C. albertensis* as full species. It appears that a similar situation could be occurring in the Palearctic. The distribution of several species passes through

drastically different biomes and habitats. Potentially, by focusing on traits like the larval habitat or elevation, a single species that is reported to be highly variable morphologically, such as *C. puncticollis*, may end up being multiple species. This highlights the need for careful and thorough field collecting for the purposes of species delimitation.

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CHAPTER IV

NEXT-GENERATION TOOLS TO CONTROL BITING MIDGE POPULATIONS

Background

Biting midges in the genus *Culicoides* are small hematophagous insects that feed on a variety of vertebrate hosts. *Culicoides* midges are responsible for transmitting over 110 viral, protozoan, and filarial pathogens worldwide [1, 2]. The diseases caused by these pathogens are of veterinary, medical, and ecological importance, and include bluetongue (BT), epizootic hemorrhagic disease (EHD), African horse sickness virus (AHSV), Schmallenberg disease, and Oropouche fever [3, 4]. Multiple outbreaks of bluetongue virus (BTV) of different serotypes, topotypes (regional variants of particular serotypes), and strains have been recorded in Europe in recent decades [5, 6]. One of the largest European outbreaks to date resulted in economic damage greater than $150 million (USD) in the Netherlands alone [7]. While severe disease outbreaks can cause a substantial loss in livestock numbers, their main economic impact stems from international trade restrictions and bans [8]. Worldwide estimates of direct and indirect losses due to just BT have been estimated to top $3 billion (USD) annually [9].

Methods of treatment and prevention for *Culicoides*-transmitted pathogens are broad and untargeted, or reactive to an outbreak, resulting in insufficient population reduction to prevent transmission [10, 11]. Current management practices for biting midges use a combination of broad-spectrum pesticide applications, larval habitat source reduction, and behavioral management of livestock [12, 13]. Implementing these strategies over a large area can be

difficult, expensive, or harmful to the environment. The availability of vaccines is also limited for many diseases caused by *Culicoides*-transmitted pathogens. Attenuated vaccines are available for BTV, though their effectiveness varies as they often only protect against a single serotype [3, 12]. Inactivated viral vaccines for BTV have also been shown to be effective, but would be expensive for large scale livestock applications in enzootic areas [14, 15]. With the concerns and limitations of the current control methods, research efforts are sorely needed to develop environmentally friendly and sustainable methods for *Culicoides* midge control.

The use of autocidal and next-generation control methods is an attractive option for implementation in *Culicoides* systems to reduce or replace natural populations and prevent disease transmission. These population-level control techniques utilize the biology of the target species to reduce the total number of vectors in a population [16, 17]. Suppression methods inhibit a target organism's ability to produce viable offspring through the release of sterile or incompatible males. This reduction in potential vectors is presumed to lead to a reduction in pathogen transmission. Conversely, methods used for population replacement aim to lower virus transmission by reducing the vector competency of individuals within the population. Replacement strategies have garnered significant attention for the control of dengue virus (DENV) transmission in the *Aedes aegypti* mosquito [18, 19, 20, 21]. Individuals resistant to pathogen transmission can be released into the environment until the disease refractory phenotype reaches fixation, thus replacing the wild population with one that has a limited ability to transmit pathogens.

Culicoides sonorensis, an important vector of BT and EHD viruses in North America, is a well-studied species with a significant number of molecular resources available making it a prime candidate for the investigation of next-generation control methods [22]. *Culicoides sonorensis* has been reliably maintained in colonies for over 60 years and existing rearing protocols can be scaled for mass production and releases [23, 24]. The genome of *C. sonorensis* is published along with several reference transcriptomic studies [25, 26, 27, 28], and further annotation and chromosomal mapping/assembly will help to maximize the utility of these resources. There are also several cell lines of *C. sonorensis* which will aid in the screening process for effector genes or *Wolbachia* strains that might interfere with pathogen replication prior to *in vivo* experiments [22]. Here we assess the potential application of autocidal, genetic-, and *Wolbachia*-based control techniques to reduce biting midge populations and as methods to limit pathogen transmission using *C. sonorensis* as a model. The outcome of initial tests within this more tractable species will help inform whether significant resources should be allocated to developing similar control methods in other *Culicoides* vector species.

Management tools

Sterile insect technique (SIT)

SIT is an autocidal, or "self-killing", approach to pest control based on the mass inundative releases of irradiated sterile males. When irradiated males mate with wild females, the lack of viable sperm transferred ultimately causes the reduction of natural populations, provided the releases are sustained [29, 30] (Figure 4.1a). SIT approaches have been used successfully to control *Cochliomyia hominivorax* (primary screwworm), *Glossina austeni* (tsetse fly), and *Ceratitis capitata* (medfly) [31, 32, 33], and are an attractive option for vector control as these

released males do not negatively impact the host via blood-feeding or by transmitting pathogens. Additionally, this approach is environmentally friendly as it is species-specific and self-limiting [34]. As an initial step towards the development of an SIT approach targeting biting midges, Jones (1967) [23] exposed males and females from the USDA "AA" colony line of *C. sonorensis* to varying amounts of gamma radiation. Sterility of 95-100% was observed in males exposed to 10,000-15,000 rad and this infertility lasted for up to 5 subsequent matings. Note: Jones lists his measure of radiation dosage as "R" which could be either rads or roentgens. Females exposed to these doses showed a drastic decrease in the number of eggs laid. Jones (1967) also demonstrated sterilization of pupae, though in many cases a higher dose (20,000-30,000 rad) was needed to prevent males from recovering fertility. A potential advantage to irradiating pupae is that fewer adverse side effects might be associated with transporting pupae as compared to the more fragile adult stages, similar to reports from shipping adult mosquitoes [35]. Even though *C. sonorensis* were exposed to relatively high amounts of radiation, little to no somatic damage was observed [23, 24]. Further studies are needed to fully investigate the use of SIT to control *Culicoides* midges; however, its simplicity and success in controlling other Dipterans makes this an promising approach.

Wolbachia-based strategies

Wolbachia is an obligate intercellular bacterium found in a multitude of insect orders and is estimated to infect up to 55% of insect species [36]. *Wolbachia* has been demonstrated to cause reproductive phenotypes in its infected hosts including male-killing, feminization of genetic males, parthenogenesis, and cytoplasmic incompatibility (CI) [36]. The most well studied reproductive modification is CI because of its applicability for insect vector control. CI results when a male infected with *Wolbachia* mates with an uninfected female or a female with a

different *Wolbachia* infection type. The result of CI is that females in incompatible crosses produce non-viable offspring (i.e., eggs that do not hatch). Low-density *Wolbachia* infections naturally occur in wild populations of several species of *Culicoides* midges in Europe, Australia, and the U.S. [37, 38, 39]. *Wolbachia* infections have also been demonstrated in several mosquito species to induce disease refractory phenotypes [40, 41, 42, 43, 44]. If *Wolbachia* strains that induce CI or pathogen refraction in their *Culicoides* hosts can be identified and transfected into important vectors, *Wolbachia*-based strategies may be a viable approach for use against *Culicoides* midges.

Wolbachia-based Incompatible Insect Technique (IIT)

Wolbachia-based IIT approaches are based upon mass releases of incompatible *Wolbachia*-infected males, which can lead to suppression and potential elimination of a localized vector population (Figure 4.1b) [45]. Like SIT, IIT also shares the same limitations of relying on consistent mass rearing and release of only males, though there is no need for specialized irradiators or radioactive materials for sterilization since *Wolbachia* induces CI. Fluorescent *in situ* hybridization experiments have shown localization of *Wolbachia* infections in the midgut, testes, and ovaries of *C. sonorensis* [39]. The localization of *Wolbachia* infections in the reproductive tracts of *C. sonorensis* is suggestive that *Wolbachia* may be influencing the reproductive system of its *Culicoides* host [46]. Furthermore, infections identified in *C. sonorensis* are in similar *Wolbachia* clades that result in CI in other insects [46]; however, *Wolbachia* induced CI or other reproductive phenotypes remain undocumented in any *Culicoides* spp. Additional studies are needed to examine for *Wolbachia* induced CI among *Culicoides* species harboring natural *Wolbachia* infections. Field and laboratory trials have shown promising results by reducing mosquito populations in several species [47, 48]. Resulting technology and

lessons learned from these studies can be used to help adapt IIT for use against *Culicoides* midges.

Wolbachia-based population replacement

Particular *Wolbachia* variants (e.g., the *w*Mel strain) partially block DENV, chikungunya virus, Zika virus, and the yellow fever virus transmission without impacting *Ae. aegypti* fitness [40, 41, 42, 43, 44]. As *Wolbachia*-infected females can mate and produce viable offspring with infected and uninfected males alike, their resultant reproductive advantage can drive a given disease refractory phenotype into a natural population (Figure 4.1d). Releases of *Wolbachia*-infected mosquitoes by the World Mosquito Programs are ongoing in 15 countries focused on reducing DENV transmission (www.eliminate.dengue.com). These releases have been remarkably successful at replacing natural populations with *Wolbachia* infected individuals and are showing reductions in DENV transmission [49]. It is presumed that these W*olbachia* infections are directly competing with the pathogens for intracellular resources or the infection is resulting in an upregulation of the host's immune system. Either or both of these could, in turn, influence the pathogen in the insect host [45, 50]. Recent transfection of *C. sonorensis* cell lines with a novel *Wolbachia* type suggest an upregulation of the host immune system, which may be associated with a pathogen blocking phenotype; however, this needs to be tested *in vivo* [51]. Population replacement approaches are an attractive option for *Culicoides* disease control as they do not require the continued release of individuals after the desired phenotype reaches fixation in a population, though this self-sustainment also increases ecological concerns. After the establishment of *Wolbachia*, restoring the natural population or eliminating the introduced population may be difficult in the event of any undesirable outcomes [52].

Transgene-based strategies

Whereas SIT elicits sterility via chromosomal damage to the reproductive cells, and certain *Wolbachia* species cause CI, infertility can also be induced molecularly through genetic modifications. Manipulating an insect's genome through the insertion of genes or altering the expression levels of existing genes can produce individuals with a desired genotype [53, 54]. Local vector populations can be suppressed or pathogen transmission can be blocked by releasing genetically modified (GM) individuals carrying a lethal or pathogen-resistant transgene [reviewed in [55]]. Autocidal or *Wolbachia*-based approaches rely on the disruption of fertilization or early embryonic development, whereas transgene-based approaches allow more control over the timing of gene expression and any associated consequences. For example, a lethal transgene can be designed to activate only during the pupal stage. This means the released GM individuals can develop normally as a larva and actively compete with wild-types for resources, potentially increasing the power of the approach. Genetic engineering can also be used in conjunction with conventional SIT or IIT [56, 57], although the designation of the organisms as GM will affect when and where this strategy can be used. Transgene-based control methods will face some of the same logistical challenges associated with mass rearing and release, but will also face public resistance due to the designation of these insects as genetically-modified organisms (GMO). In fact, the use of GMOs are outright banned in some countries, a situation unlikely to change in the foreseeable future.

Currently, methods for the genetic modification of *Culicoides* midges have not yet been described; however, tools such as CRISPR-Cas9 [58] and the broadly active transposable element *piggyBac* [59] are likely to be effective in biting midges, given their success in many other Diptera. Once validated methods are in place, genetic modifications proposed as a means to

control other vector populations such as mosquitoes can be used as a template to create similar strains of transgenic *Culicoides* midges [60, 61]. Additionally, without a phylogeny for the genus, it will be hard to predict what information will be transferable between or with subgenera. As gene families can evolve independently between divergent groups, the genes associated with pathogen transmission or midge reproduction/development may be highly variable within the genus. Separate transgenic suppression or replacement methods may need to be developed for specific midge species, further complicating this approach.

Self-limiting transgene-based population suppression

Genetic control techniques such as RIDL (Release of Insects carrying a Dominant Lethal) can be adapted for use in *Culicoides* [55] (Figure 4.1c). Males carrying a dominant lethal transgene are mass-released into a population with all of their progeny inheriting a copy of this transgene. The female offspring will subsequently die; however, the male offspring will survive and pass this transgene to 50% of their progeny offering multigenerational control. To maintain transgenic lines within the production facility, this approach requires the development of an inducible sex-lethal system to turn off expression of the lethal gene, similar to the Tet-on/off [62]. These lines can also be integrated into an SIT or IIT approach as a means of improving the speed and accuracy of sex-separation.

Transgene-based population replacement and gene drive

Certain *Wolbachia* spp. are capable of overcoming normal Mendelian inheritance with the result that these strains can increase in frequency in the host population without actually offering a benefit or selective advantage. Similar methods of increasing transgene frequency in wild populations have been proposed to spread engineered transgenes to be used in population

replacement approaches; these are termed gene drive [reviewed in [63]]. A wide array of gene drive architectures have been developed in other Diptera such as *Drosophila* [19, 64, 65] and mosquitoes [66, 67, 68, 69]. These will be helpful for the development of any future *Culicoides* midge population replacement strategy as the general principles of pathogen resistance and gene drive will be the same [50, 70, 71] (Figure 4.1d). The effects of natural *Wolbachia* infections in target *Culicoides* species on a gene drive approach could also be nullified by the development aposymbiotic strains via antibiotic treatments. Any population replacement strategies developed using a gene drive system can be expected to vary in terms of persistence and invasiveness in the environment, and thus proper risk assessment and community engagement will be vital before any field-based evaluation or implementation [45, 72].

Research gaps concerning novel control approaches against *Culicoides*

Investigation of sterility, CI, genetic modification, and pathogen blocking phenotypes

The next-generation management techniques mentioned above rely on the creation of a targeted phenotype to then be released into natural populations. The mechanisms underlying these approaches all vary; however, each will follow similar steps during development (Figure 4.2). Solutions for overcoming hurdles in one strategy will likely translate to the others. Mating assays are often used to test the efficacy of a created phenotype measuring clutch size, hatch rate, immature survival, and inheritance [45, 67]. This can only be done if the target species mates under laboratory conditions and many *Culicoides* spp. form mating swarms at established landmarks (environmental structures, larval habitats, the host, etc.) [73]. As colonies of *C. sonorensis* exist, laboratory mating assays should not impede the development of next-generation control strategies against this species.

Further work is needed to refine the radiation sterilization of *C. sonorensis* using modern methods and equipment, but this work can be started immediately. To introduce a *Wolbachia* strain or perform genetic modifications, a protocol for the microinjection of biting midge eggs must be established. While there are well-established protocols for microinjecting mosquito eggs [74], *Drosophila,* and sandfly eggs [75], *Culicoides* eggs are more elongated and have less volume, and are thus anticipated to be more difficult to manipulate (Figure 4.3). Until successful microinjections can be conducted, this will be a barrier to the implementation all *Wolbachia*- and genetic-based strategy.

In developing new protocols for genetically modifying biting midges, we anticipate that the initial modifications would be the insertion of a marker gene such as green fluorescent protein (for transposon-based approaches) or easily scorable visible markers such as white-eyes (for CRISPR/Cas9 based approaches) [76, 77]. Following the validation of injection methods and transposable element (TE) integrations, the TE-based random insertion of candidate transgenes will help determine optimal integration site [78]. Such sites could be re-used using targeted recombinases [79], or using CRISPR-Cas9 [80]. Research suggests that BTV vector competence is associated with the expression of glutathione S transferase (gst) and the antiviral helicase (ski2) [28, 81], thus altering the expression of these two genes with inserted promotors or suppressors may be a first step towards a genetic-based control strategy.

Many different cell lines derived from *C. sonorensis* exist and their susceptibility to a number of BTV and epizootic hemorrhagic disease virus (EHDV) serotypes are known [22]. Additionally, cell lines derived from *C. nubeculosus* have recently been established [82]. As an initial step to investigate virus inhibitory effects induced by *Wolbachia* infections or transgene-based genetic modifications, the rate of viral proliferation in modified cells can be compared to

previous studies. If inhibition is found, running these assays on lines from both species will aid in understanding the mechanisms behind this inhibition.

Establishment of laboratory colonies and the logistics of mass release

For SIT, IIT, or GM suppression methods to be effective, the target biting midge species must be continuously mass-reared on the scale of tens of thousands of individuals. Whereas replacement strategies do not require inundative releases, they still require the repeated release of substantial numbers of individuals proportional to the natural population. *Culicoides sonorensis* is one of two species of *Culicoides* currently maintained in colonies. Initial colonization of wild-collected *C. sonorensis* can be difficult as well, but there are procedures and protocols in place to aid any attempts [23, 24]. The number of individuals currently being produced (2 million per year) is only constrained by space and labor and can be scaled upward as needed. The colonization of other *Culicoides* species has been attempted, but to date, these efforts have been met with limited success or the discontinuation of colonies [22, 83]. The inability to successfully colonize and maintain a species in the laboratory would render these control methods ineffective for that species, making this an urgent area of research need.

For example, the subgenus *Avaritia* contains several primary vector species of pathogens associated with livestock disease on four continents [2]. Two of the most significant vectors in Europe are *C. imicola* and *C. obsoletus*, which transmit BT and Schmallenberg viruses [3]. *Culicoides imicola* is also a significant vector in Africa where it transmits AHSV. Members of this subgenus feed primarily on a variety large mammals and have the ability to breed in dung; tightly linking their life history with susceptible hosts [84]. Attempts to produce viable offspring from field collected *C. imicola* and *C. obsoletus* have resulted in high oviposition numbers and

hatch rates, but high larval mortality [85, 86, 87]. Optimization of larval rearing conditions could increase the overall adult yields. Interestingly, results from these studies show an apparent male sex-bias from lab reared individuals. The mechanisms behind this are unknown and this may present an additional hurdle in the colonization of these species. Potentially, successful control of one *Culicoides* species using next-generation management tools will signify that substantial resources should be invested in developing and maintaining colonies of these, or other currently intractable midge species of substantial veterinary importance.

Sex separation technologies

Sex separation can be a bottleneck in the workflow of mass-rearing insects for inundative release, and for certain approaches, it is vital for continued efficacy [30]. In autocidal and genetic-based suppression methods, the unintended release of females will not affect the overall efficacy of the control strategy. With IIT however, release of *Wolbachia*-infected females could spread the infection into the natural population, nullifying any CI from that strain. There are clear sex differences in *C. sonorensis* apparent in the adult and pupal stage [88, 89], though separating the sexes manually is labor-intensive. Sex separation in mosquitoes can be done manually, mechanically, genetically, with insecticide-laden blood meals, or with machine vision technologies [90, 91, 92, 93]. Many of these techniques could be modified for use in *Culicoides* midges and evaluated for efficacy and accuracy. Additionally, removal of the females at the adult or pupal stage wastes resources that could be better spent increasing male production. Currently, genetic engineering is the only method that could be used to remove female mosquitoes as larvae. This can be done using a Y-linked fluorescent or visible marker [94, 95], or with conditional sex-lethal genes [62, 96, 97, 98]. The remaining males can then be conventionally sterilized, released carrying a transgene, or released infected with *Wolbachia*.

Examination of life history traits and fitness

Life history traits also need to be considered and investigated for autocidal, genetic, and *Wolbachia*-based approaches. For example, the number of times females will mate can impact the efficacy of certain methods. Infertility caused by mating with sterile males can be undone by a single mating with a wild-type male. Additionally, traits such as longevity, survivorship, fertility, and fecundity, can be affected by lab rearing insects [93, 99, 100]. The release of males that are less fit and less competitive will subsequently reduce the success of these approaches. Female fitness influences the rate at which a *Wolbachia* strain or transgene will spread in natural populations as part of a replacement approach. Future work needs to include investigating the fitness of individuals from all of the aforementioned strategies in lab and field conditions.

Vector-Host-Pathogen Interactions

The majority of the economically important *Culicoides*-transmitted viruses can be transmitted by multiple species, and there are likely more vector species yet to be identified [1]. Disease causing pathogens have been isolated from a number of *Culicoides* spp., though dissemination and oral transmission need to be demonstrated in many of these cases [2]. Disentangling incidental infections from the species most important for maintaining transmission should be a priority. Doing so will help assess the practicality of using next-generation control techniques against biting midges and identify where they are most likely to succeed. As multiple vector species can occur sympatrically, the management of a single species may not be sufficient to eliminate or possibly even reduce pathogen transmission. The predominant vector species can also change by region; therefore, these species-specific control measures will be limited in their use geographically. However, even regionally managing virus transmission can reduce the risk of

incursions into disease-free areas. Finally, in the event of an exotic virus introduction, such as African horse sickness, having additional tools to use in tandem with pesticides and quarantines will be important.

Regulatory Approval

The use of SIT for control of insects is bound by few regulatory hurdles in the U.S. and internationally. Currently, there are no international agreements or regulations on the commercial production and release of sterile insects. That being said, the irradiation procedure is becoming more difficult, with reported delays and denial of shipments of Cobalt-60, the common material used for small scale irradiators. Irradiation using isotopes is subject to federal approval in the U.S. and the International Atomic Energy Agency. The use of X-ray radiation to sterilize insects for pest management approaches can address some of the programmatic issues of irradiation procedures. Small scale X-ray irradiators require less shielding and precautionary measures, are easy to use, are portable, and only require an electrical power source [101]. *Wolbachia* approaches are currently regulated in the U.S. by the Environmental Protection Agency (EPA). Only one IIT approach, for the mosquito *Aedes albopictus*, has been approved to date for commercial sales in the U.S. [47]. Other IIT and *Wolbachia*-based replacement approaches have been approved for use in multiple countries [49, 102, 103]. Large-scale releases of GM mosquitoes have been carried out in Brazil, Panama, and the Cayman Islands [104, 105, 106], and EPA has issued an experimental use permit for releases in Florida and Texas [107]. Regulatory approval for using *Wolbachia*- and genetic-based approaches are at the purview of each country performing the releases and could require additional approval from a local governing entity.

Semi-field and small scale field trials

Information on the short-range dispersal of *Culicoides* midges will be useful in determining localized effectiveness of these strategies. Both the males and females of several *Culicoides* species can disperse 1-3 km, both upwind and downwind in only a few nights [108, 109]. Male swarms will form near the larval habitat or at a swarm-marker [110, 111]; however, in the case with *C. sonorensis*, mating occurs on or near the host [73]. As the males of this species will move to the host, one centralized release point is likely enough to cover a large area for population suppression. In regards to long-range dispersal, *Culicoides* midges possess the ability to disperse via jet streams and thus have the potential to establish long distances from a release site [112, 113]. This behavior will need to be considered during risk assessment of population replacement strategies. Small cage and semi-field trials will also need to be performed for the suggested control approaches, and the work done with mosquitoes can be used as a template in studying *Culicoides* midges. These trials will determine the efficacy of each approach and act as proof of concept by gaining an understanding of fitness effects, mating competitiveness, survival, and rates of sterility and/or replacement.

Implementation

Though many species of *Culicoides* are associated with disease transmission, here we will highlight two systems for which autocidal and next-generation approaches could be applied; population suppression of *Culicoides belkini* and population replacement of *C. sonorensis*. Some species of biting midge species, such as *Culicoides furens* in the Caribbean and *C. belkini* in the south Pacific, are not disease vectors but do adversely impact outdoor activities and tourism resulting in severe economic impact to the island economies [114, 115, 116]. *Culicoides belkini*

populations are excellent targets for population suppression because the isolated island distribution likely limits migration between islands. Institute Louis Malarde recently completed a 600 square meter rearing facility to raise *Wolbachia* infected *Aedes polynesiensis* mosquitoes for population suppression on the Society islands. This same facility can be used to first colonize and then mass rear the native *C. belkini* (personal communication with the Lab Director Herve Bossin). Local populations or small islands can be targeted to prove efficacy of the population suppression or elimination, which will have significant local support by the community and tourist industry. *Culicoides belkini* is the only species of biting midge on certain islands, therefore monitoring the population reduction and detecting reintroductions can be coupled with the local *Ae. aegypti* and *Ae. polynesiensis* monitoring program with relative ease.

Eliminating or reducing a *Culicoides* midge population below a theoretical transmission threshold may not be feasible on a continental scale compared to isolated islands. The alternative is a population replacement strategy to reduce a species vectoral capacity. For BTV, EHDV, and vesicular stomatitis virus (VSV) in the United States, the best known vector is *C. sonorensis*. This species native range is the central and western United States, primarily west of the Mississippi river [10]. Releasing *Wolbachia*-infected or transgenic *C. sonorensis* with reduced vector competence will not eliminate the population but could help reduce overall virus transmission, even in the presence of other competent vector species [117]. While transmission may not be completely abrogated due to the presence of these other species, pre- and post-release serosurveys of sentinel animals can be used to document any reduction in pathogen transmission [118, 119]. This in turn can shed new light on the importance of *C. sonorensis* in driving transmission of BTV and EHDV, and help identify other vectors that might be important in this process. Moreover, the range of *C. sonorensis* is most of the US west of the Mississippi river,

and so, releasing fewer individuals (compared to inundative releases) is desirable due to the probable high effective population size as has been shown in *C. imicola*, *C. obsoletus*, and *C. brevitarsis* [120, 121, 122]. Although the ultimate goal is to eliminate viral transmission, the initial goal of next-generation management practices is to prove their effectiveness against biting midges. Again, success or failure with any approach like this can help inform whether similar approaches will be of value for other *Culicoides* spp.

Engagement and risk assessment

Stakeholders

Though hemorrhagic disease (HD) caused by BTV and EHDV is usually subclinical or asymptomatic in goats and cattle, it can cause severe symptoms and subsequent death in deer and sheep [123, 124]. These diseases cost the United States roughly 125 million USD annually [125] and this likely underrepresents the current economic loss as seen in more recent estimates of the global impact of BT [126]. Commercial deer breeding is the most heavily impacted livestock industry in North America, and in Europe, sheep are most the most susceptible [127, 128, 129]. Thus, in regards to biting midge control efforts, these farmers are likely to be a primary stakeholder for the use of novel control approaches, especially during disease outbreaks. The organization and structure within these industries will prove beneficial to community engagement efforts to promote novel control approaches at annual meetings and though farmer associations. Secondarily, the cattle and dairy industries have a financial interest in reducing the transmission of these viruses. Though these animals are asymptomatic, trade restrictions and reduced production are still associated with HD [126]. Farms and ranches are often on large plots of privately-owned land, away from cities and towns. The release of sterile or modified males on

these farms can offer localized management while maintaining a comfortable distance from the general public. Community-wide surveys and collaboration with local governments in these areas will determine if the isolation of these releases increases their overall acceptance.

Risk Assessment

Autocidal, genetic-, and *Wolbachia*-based methods for vector control can be self-limiting or self-sustaining and the risks associated with each should be weighed alongside any potential benefits prior to release [34, 52, 72]. Proper ecological risk assessments of most species of *Culicoides* midges will be challenging, as certain biological traits remain unknown. For example, of the roughly 1350 species worldwide, the immature stages have been described for less than 20% of the species [112]. For the species that have been described, there are limited diagnostic characteristics available and identification can be difficult or inaccurate. Surveying larval habitats is less important for SIT or IIT, but some genetically engineered control strategies employ this step in risk assessment and monitoring.

For control methods that rely on sterility or incompatibility, heterospecific mating will not affect the outcome of the program; however, for methods that rely on a drive mechanism, gene flow between closely related species can have unintended consequences. There are a number of species complexes within the genus that hinder proper identification and depending on the relatedness of the species within the complex, could lead to unintended consequences upon release of modified individuals [84]. *Culicoides sonorensis* belongs to a complex of three species, which was historically considered five subspecies [89]. If *C. sonorensis* is actively hybridizing with closely related species, the risk of introgression increases substantially. Under laboratory conditions, *C. sonorensis* was able to hybridize with *C. occidentalis* and produce

viable offspring for six generations [130]. In nature, pre-zygotic isolation barriers can still exist to keep these species from mating; however, this study shows the potential for gene flow due to the lack of post-zygotic isolation barriers. No natural hybrids have been confirmed, though analyses using more sensitive markers should be conducted [131, 132]. As monitoring programs will be in place to ensure the efficacy of a management program, these can also be used to detect unintended outcomes from releasing modified individuals into the environment.

Conclusions

As novel strains of *Culicoides*-transmitted viruses continue to spread to new areas [133, 134, 135], establishing one or multiple next-generation control methods could provide an effective way to reduce disease transmission. Successful control in one species would provide an outline to adapt these techniques for use in other biting midge pathosystems. Conventional SIT can be the most turnkey option for controlling biting midges, though more work is needed to optimize the radiation dose to minimize any fitness effects attributed to irradiation. Evidence for CI induced by *Wolbachia* infections in *Culicoides* is still needed, but both IIT and *Wolbachia*-based replacement approaches appear promising. No genetic modification of any *Culicoides* midge has been reported; therefore, its use in vector control is likely years away; although developing conditional sex-lethal transgenic lines could be useful for integration of an SIT or IIT approach. Any potential *Wolbachia*- or genetic-induced viral inhibitory effects will need to be demonstrated using *in vitro* and *in vivo* systems. Although this review focused mainly on North America and *C. sonorensis*, there are a multitude of *Culicoides* systems that can benefit from these next-generation control techniques. Each of these systems will have its own challenges and hurdles to consider before implementation; however, the ability to preemptively apply the

knowledge gained from researching *C. sonorensis* will be invaluable to adapting these tools for use against other biting midge species.

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Figures

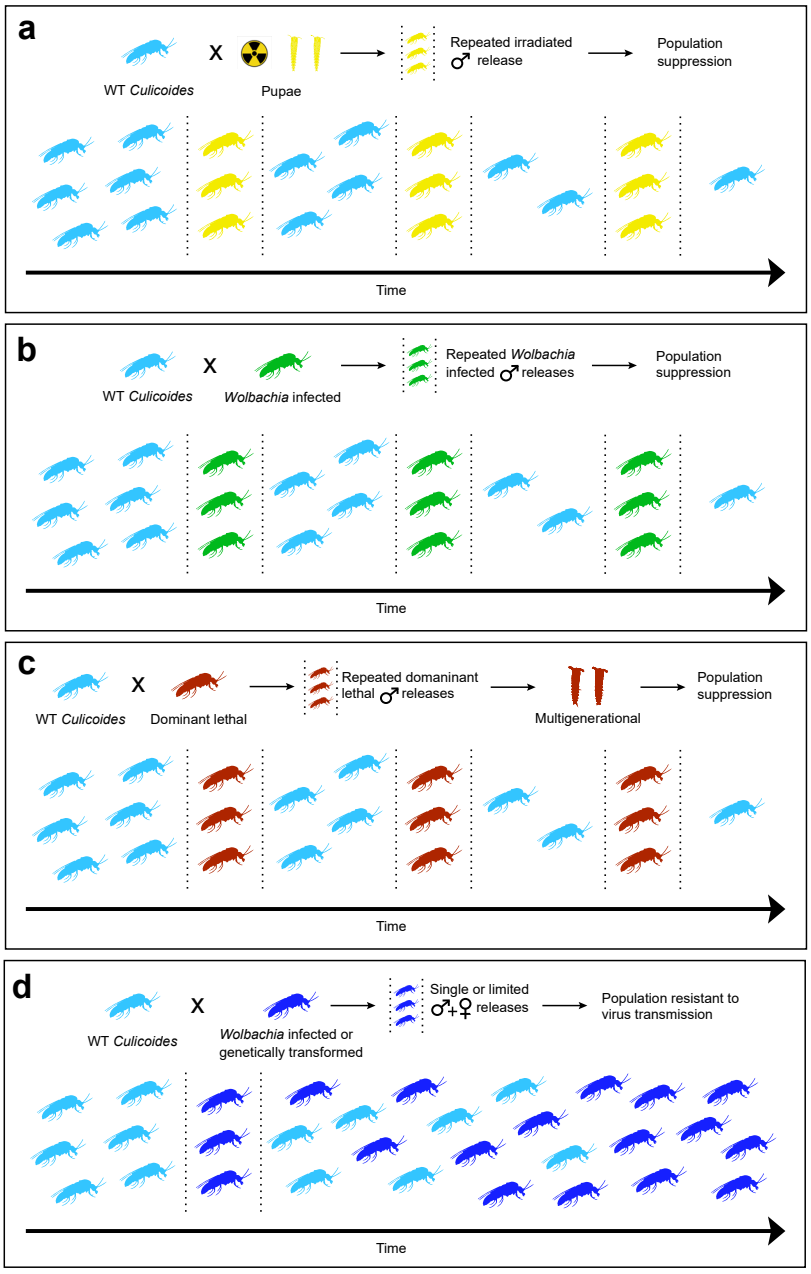

Figure 4.1. Proposed suppression and replacement approaches for *Culicoides* population and disease control: (**a**) Sterile insect technique (SIT) approach, (**b**) *Wolbachia*-based IIT approach, (**c**) male dominant lethal population suppression, and (**d**) *Wolbachia*-based population replacement and genetic modification gene drive approaches. In all figures the light-blue *Culicoides* midge symbols represent wild type individuals.

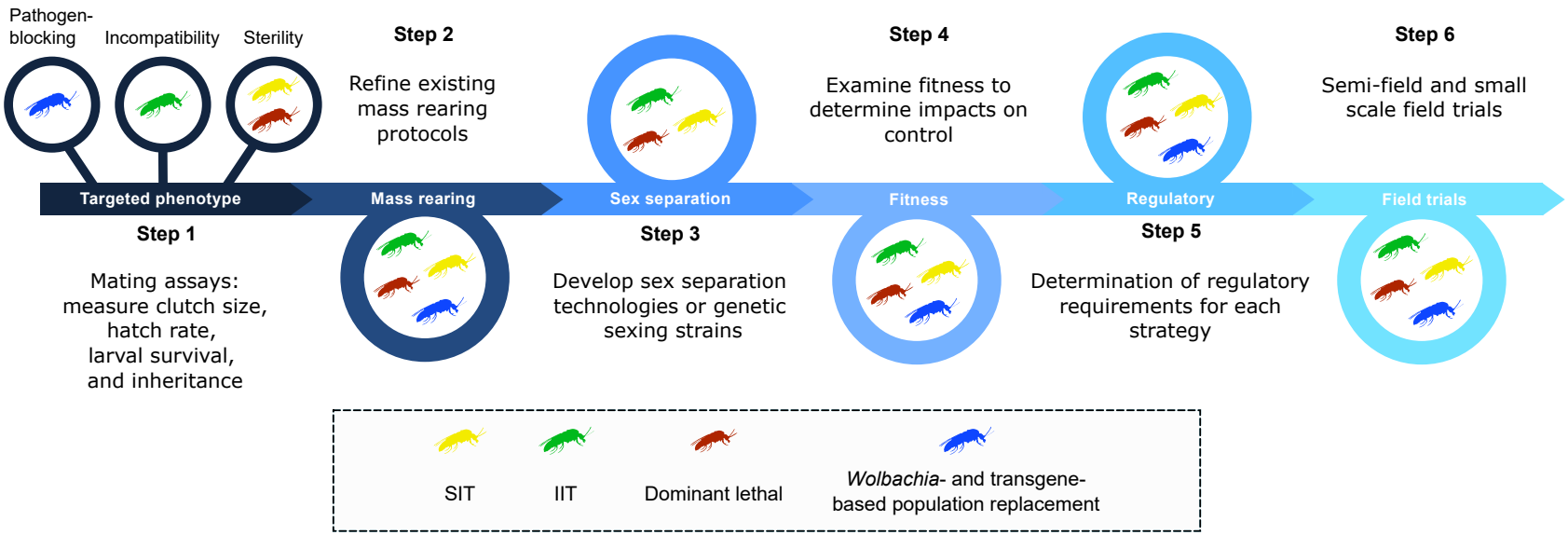

Figure 4.2. Systematic steps for future research to address hurdles for the development of IIT, population replacement, genetic based, and/or SIT approaches.

Figure 4.3. Side by side comparison of *Culicoides* eggs compared to eggs of other Diptera. Species eggs are displayed from top to bottom: *C. sonorensis*, *Aedes albopictus*, and *Drosophila melanogaster*, respectively. The image was taken at 40x magnification.

CHAPTER V

CONCLUSION

Before this study, only three species were recognized within the *C. variipennis* complex. Here, I present evidence that suggests this is an underrepresentation of the true number of species within this species complex. SNP data obtained from individuals of the *C. variipennis* complex across the US and Canada revealed clear delineations between five species, even within sympatric populations. In addition to *C. occidentalis*, *C. sonorensis*, and *C. variipennis*, these data support the raising of *C. albertensis* to full species status and the description of the new species *C. mullensi*. Morphological analyses and bionomic study provided further support for these taxonomic assignments as well as the raising of *C. australis* to full species status. The species boundaries inferred from the SNPs were clearly delineated; however, the mitochondrial data were more ambiguous. Without the species identification of the SNPs, I would not have been able to accurately interpret the mt data. The COI haplotype network showed significant geographic divergence between populations of *C. occidentalis*, while almost no divergence was seen among *C. albertensis*, *C. sonorensis*, and *C. variipennis*. Though the causes of this are unknown, it at least explains why we have encountered so many issues with species delimitation in the *C. variipennis* complex using the COI gene. Fortunately, the data collected in chapter II should allow for the development of a molecular diagnostic tool for use in vector surveillance.

A significant portion of the subgenus *C.* (*Monoculicoides*) was revised in this study. In addition to the changes made within the *C. variipennis* complex, evidence of another derived clade within *C.* (*Monoculicoides*), the *stigma* group, was supported. Morphologically, this group contains characters that appear to be unique within all of Culicomorpha. Further study of the character states within this subgenus may provide evidence of the phylogenetic position of these groups. As the monophyly of *C.* (*Monoculicoides*) is well supported, certain biological patterns

highlighted here are most likely derived. The females of each species within this subgenus feed primarily do so on large mammals (Table 5.1). As such, some species are known to breed in habitats contaminated by the feces of these hosts, but there are multiple other habitats to which species within this subgenus have become adapted. Additionally, there are differences in mating behaviors, which in the *C. variipennis* complex at least, appear to be maintaining species boundaries. Further phylogenetic analysis of this group is needed to interpret these biological traits from an evolutionary standpoint. The more monophyletic clades that can be identified and analyzed in this way, the more complete picture we will have of the overall diversification of *Culicoides*.

Table 5.1. The known bionomic information of the species within *C*. (*Monoculicoides*).

Species	Distribution	Larval habitat	Host	Mating at	Vector species
albertensis	Nearctic	alkaline	vertebrates	?	?
australis	Nearctic	saline	vertebrates	larval habitat	no
combinothecus	Palearctic	?	vertebrates	?	?
cornutus	Afrotropical	contaminated water	large mammals	larval habitat	no
digitalis	Palearctic	fresh water	vertebrates	?	?
expallens	Palearctic	fresh water	vertebrates	?	?
grandensis	Nearctic	alkaline	none	?	no
heiheensis	Palearctic	?	vertebrates	?	?
helveticus	Palearctic	eurytopic	vertebrates	?	?
homotomus	Oriental	contaminated water	large mammals	?	no
lochmocola	Palearctic	?	vertebrates	?	?
longicollis	Palearctic	saline	large mammals	?	?
longlinensis	Palearctic	?	vertebrates	?	?
mullensi	Nearctic	saline	vertebrates	?	?
nanpingensis	Palearctic	?	vertebrates	?	?
nubeculosus	Nearctic	contaminated water	large mammals	host	no

occidentalis	Nearctic	saline	large mammals	larval habitat	no
pachynonus	Afrotropical	?	vertebrates	?	?
parroti	Palearctic	eurytopic	large mammals	larval habitat	no
puncticollis	Palearctic	contaminated water	large mammals	host	no
riethi	Holarctic	saline/alkaline	large mammals	larval habitat	no
shemanchuki	Nearctic	alkaline	none	larval habitat	no
sonorensis	Nearctic	contaminated water	large mammals	host	yes
stigma	Holarctic	eurytopic	large mammals	?	no
taonanensis	Palearctic	?	vertebrates	?	?
variipennis	Nearctic	contaminated water	large mammals	larval habitat	no

Note: the host listed as "vertebrates" is deduced from the presence of fine mandibular teeth of the female adult, with no actual observations made of their specific hosts.

As some species of *Culicoides* are pathogen vectors, a majority of the work on this group is focused on epidemiology, surveillance, and control efforts. Each of these has its own set of challenges, but perhaps none greater than control. Several reviews have pointed out the limited number of options available for the control of biting midges and by far the most common method used is the application of broad-spectrum insecticides. An improved understanding of the molecular basis of the biology and epidemiology of biting midges can be leveraged in the development of next-generation control strategies. Both *Wolbachia* and genetic-based control methods are being field tested for the suppression of mosquito populations. The largest hurdles to overcome for the use of these against biting midges are the logistics of consistently maintaining a large number of insects in colony and the manual labor associated with sex-sorting, release, and monitoring. As we are presently unable to maintain certain *Culicoides* vector species in colonies, the use of population suppression methods is limited. Population

replacement strategies would need to be developed essentially from scratch, as no molecular modification of *Culicoides* has been attempted, but the dispersal capabilities of biting midges could allow for the spread of a target gene over a large geographic area. Proper ecological risk assessment would be vital to the approval of this type of control effort. It would appear that *C. sonorensis* would be the easiest species in which to test the feasibility of population-level control methods, though these methods might be most effective in island settings against species such a *C. belkeni*. Each species of *Culicoides* will have its own issues to contend with, but having more ecologically friendly and sustainable options to protect livestock may be advantageous.

Future work

The lack of population structure and high dispersal capabilities of *Culicoides* biting midges provides an interesting system in which to study speciation. Most *Culicoides* species maintain a relatively high level of gene flow between populations, even at great distances, and wind-mediated dispersal is the primary mechanism proposed to allow for this to happen. As wind dispersion is present in all basal lineages of Ceratopogonidae, the diversification of *Culicoides* took place in the presence of this ability. This could hinder geographic isolation and allopatric speciation therefore; ecological divergence may be contributing to the diversity within this group. Evidence for this is present in the *C. variipennis* complex as the speciation of this group is relatively recent yet only two of the six species share a larval habitat.

While the species boundaries of the *C. variipennis* complex appear stable, several hybrid individuals were detected and this could be due to semi-permeable reproductive barriers. Ongoing hybridization may have caused some of the patterns observed in the COI data, but in order to assess this further, the possibility that this is caused by the sequencing of pseudogenes

must be ruled out. As a preliminary step to testing if the patterns observed in chapter II extended to the rest of the mitochondrial genes, I sequenced the mitogenome of several species of *C*. (*Monoculicoides*). Additionally, further sequencing of these individuals allowed for the creation of a new SNP dataset, and both were used to construct phylogenetic trees. The same levels of convergence and divergence within the *C. variipennis* complex were found using all 13 mt coding genes as were found using the COI gene alone (Fig. 5.1). This is strong evidence that the COI sequences obtain in chapter II were not pseudogenes and that these patterns must have been caused by biological processes.

The phylogenetic trees produced by both the SNP and mt datasets agree upon the monophyly of the *C. variipennis* complex with relatively shallow divergence. However, the relationships outside of this group vary considerably, and in many cases, with high bootstrap support in both trees. The mt tree suggests that a clade containing *C. homotomus* is the sister to the *C. variipennis* complex and that *C*. *riethi* is the most basal species of *C*. (*Monoculicoides*). Other than a few superficial morphological similarities, nothing would support these relationships. The SNP tree on the other hand, has the rest of *C*. (*Monoculicoides*) separated from the *C. variipennis* complex with *C. puncticollis* as the most basal lineage. This would require that the plesiomorphic condition of this subgenus to be an elongate spermatheca, or for this character to be homoplastic. The positioning of *C. parroti* (a representative taxon of the *stigma* group) between these two trees is also not in agreement. While both suggest that this is a derived clade, there is almost no morphological support that *C. puncticollis* is closely related to the *stigma* group relative to the rest of the subgenus. *Culicoides riethi* is also quite different morphologically from the *stigma* group, however, the more ovular shape of this species' spermatheca is at the very least more similar to what is seen in *C. parroti*. This is merely

conjecture though and with such different topologies, only analysis of the biological features within the phylogenetic context of these trees will aid in the assessment of their phylogenetic signal. However, determining which of these methods produces more accurate results within this group will be invaluable to a large scale and much needed phylogenetic study on *Culicoides* as a whole.

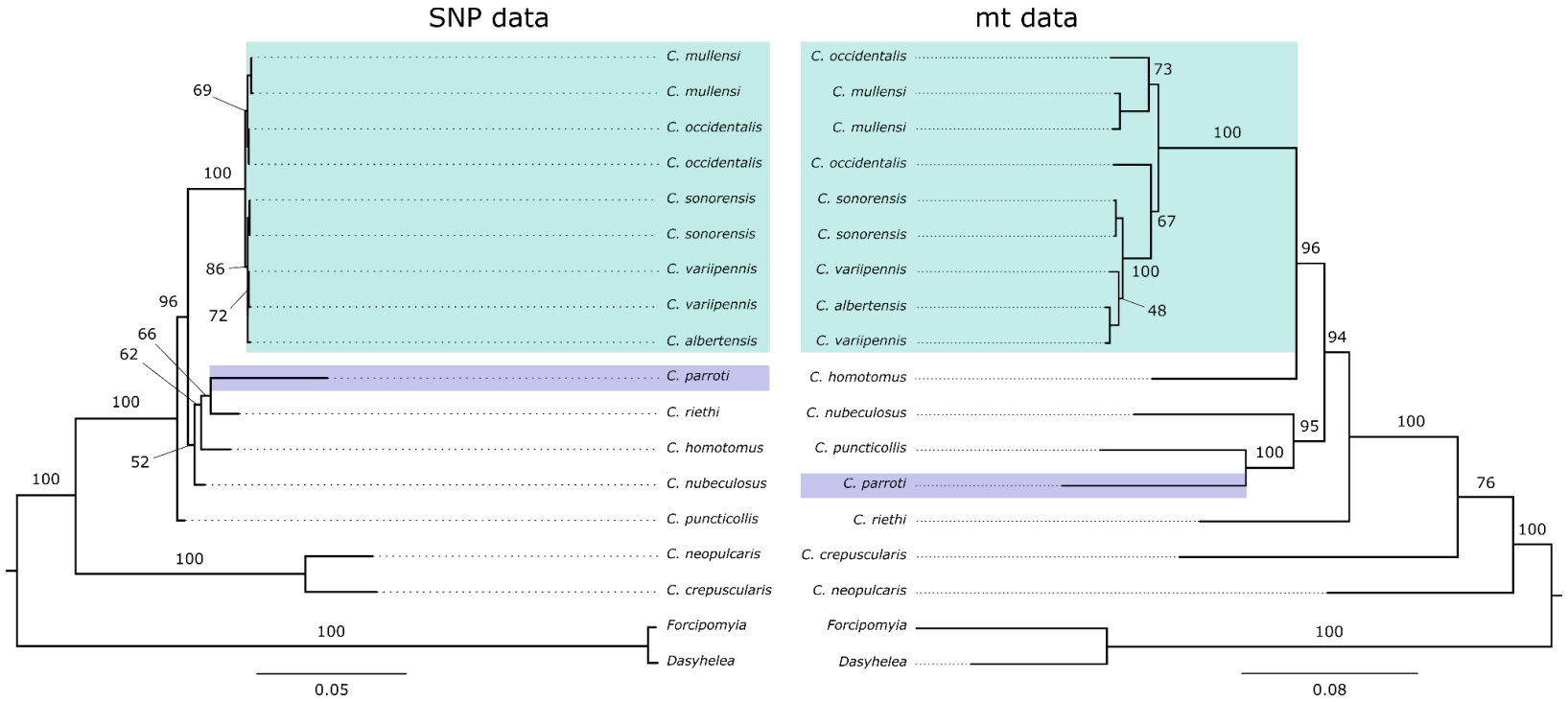

Figure 5.1. A comparison of maximum likelihood phylogenetic trees constructed using SNP data (left) and mitochondrial data (right). The green box is used highlight the *C. variipennis* complex and the blue box shows a species of the *stigma* group.