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Preliminary Analysis of Population Differentiation of Culex tarsalis in Montana Using Microsatellites

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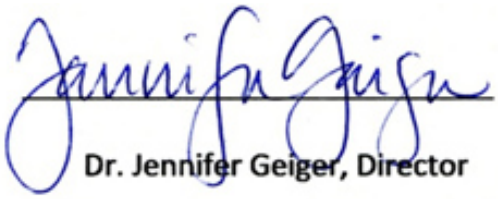

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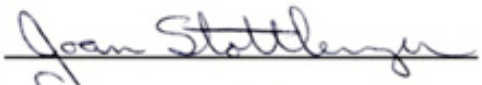

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Abstract

West Nile virus, an arbovirus classified in the genus *Flaviviridae*, presents a serious threat to humans and horses in Montana. The virus, first isolated in the West Nile district of Uganda in 1937, spread rapidly across the United States after its initial introduction to New York in 1999. In 2003 there were 222 human cases of West Nile in Montana. The overarching goal of the Carroll College West Nile virus study is to create a risk assessment map for the state of Montana. This will bring better awareness to the risk of West Nile infection based on geographic location and time. The specific goal of this project was to use microsatellite data to assess the genetic differentiation of the mosquito vector, *Culex tarsalis*, between study sites across the state of Montana, and to evaluate if populations separated by greater geographic distance have higher F_{ST} values than those located closer together. Multiplex and individual locus PCR methods were used to amplify five microsatellite loci. H_E (heterozygosity) values for each population were obtained, and F_{ST} values were obtained for between-population comparisons. My hypothesis that F_{ST} values increase as geographic distance increases was not supported. Harlem and Medicine Lake had a lower F_{ST} than Medicine Lake and Bowdoin, which are separated by a smaller geographic distance. Three factors – small sample sizes, high frequencies of null alleles, and deviations from Hardy- Weinberg equilibrium – are almost certainly confounding the data, making meaningful conclusions largely unfeasible to draw.

Introduction

Background

West Nile virus (WNV), first characterized in Uganda in 1937, became known as a severe meningitis- and encephalitis-causing disease after an outbreak among the elderly population of Israel in 1957 (Center for Disease Control (CDC), 2004). By 1999 the virus had reached North America (CDC, 2004). Between 1999 and 2001, 149 cases were confirmed by the CDC, including 18 human fatalities (CDC, 2010). WNV moved from the suburbs of New York down the Eastern Seaboard and underwent an impressive range expansion in 2002. It became the nation's largest epidemic of a neuroinvasive arboviral disease (McDade, 2004). WNV was detected in Montana by 2002 with two reported cases (CDC, 2002), and in 2003 Montana reported a staggering 222 cases of the 9862 cases nation wide (CDC, 2003).

WNV can cause serious human and equine illness. The CDC (2006) reports that symptoms are flu-like and include the following: fever, nausea, headaches, vomiting, body aches and occasionally swollen lymph glands and a rash on the chest, stomach and back. Of the 20% of people who experience symptoms when infected, one in 150 individuals will show more severe symptoms: high fever, headache, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, vision loss, numbness, paralysis and even death. Permanent

neurological effects are possible for those individuals with more severe symptoms.

WNV is an arbovirus classified in the genus *Flaviviridae* and is an affiliate of the Japanese Encephalitis (JE) virus serocomplex (Bertolotti *et al.,* 2006). WNV is largely transmitted in the western United States by the mosquito vector *Culex tarsalis* (Diptera: Culicidae) (Goddard *et al.,* 2002; Reisen *et al.,* 2004; Venkatesan *et al.,* 2010). *Cx. tarsalis* transmits the virus both vertically (generation to generation) and horizontally (orally) allowing it to overwinter and persist into the next breeding season, and it is one of the most efficient organisms at transmitting the virus of the 60 West Nile virus vectors known (Goddard *et al.,* 2002, 2003).

Cx. tarsalis plays a crucial role in the enzootic cycle of WNV (CDC, 2010). The virus is transmitted from bird to bird via the enzootic bridge vector (mosquito) and resides in various reservoir bird species (CDC, 2010). The female mosquito transmits the virus when it takes a blood-meal from an infected host (CDC, 2010). The virus undergoes primary amplification in the salivary glands of the mosquito before it bites and infects another host (CDC, 2010). Birds are the primary host for *Cx. tarsalis*, but the mosquitoes feed on mammals in late season if the opportunity arises (Reisen and Reeves, 1987). Mammals are a dead end in the enzootic cycle, however, because the human viremia is not high enough to be transmitted by a mosquito taking a blood-meal (CDC, 2006).

Variation in Vector Competency

Though little is known about the relationship of population structure of *Cx. tarsalis* to variation in vector competency, QTL (quantitative trait loci) studies of *Aedes aegypti* and dengue virus have shown a significant relationship between geographic variation of the mosquito and disease incidence (Black *et al.,* 2002). Variation in the ability of *Cx. tarsalis* to efficiently transmit the virus has been documented (Goddard *et al.,* 2002, 2003). It is possible that the geographic variation (and genetic variation) in *Cx. tarsalis* may play a role in vector competency and thus human risk of WNV exposure.

The Utility of Microsatellites

Microsatellites are molecular markers useful for characterizing population structure because they theoretically allow variation to be detected without linkage bias because they are regions of DNA that are not protein coding, and therefore are assumed to be neutral with respect to fitness. Studies ranging from *Cacao* (chocolate) to mosquitoes have utilized microsatellites to answer ecological questions relating to population structure and gene flow (Selkoe and Toonen, 2006). Microsatellites allow researchers to perform ecological studies on a fine scale better than almost all other genetic markers (Selkoe and Toonen, 2006) and allow detection of relatively low rates of migration to be distinguished from panmixia, in which there are no barriers to genet flow between populations. (Venkatesan *et al.,* 2007b). Microsatellites are

more useful and efficient than allozymes, ISSR, RAPD, SNP and AFLP methods because they require less sample preparation and have higher information content (Selkoe and Toonen, 2006). Microsatellites mutate rapidly and are thus useful for relatively short-term studies where rapidly mutating neutral genes are difficult to identify (Selkoe and Toonen, 2006). Homoplasy in microsatellite analysis, arising when characters are incorrectly assumed to be inherited from a common ancestor, is expected to be relatively low in most cases except between extremely distantly related populations (Selkoe and Toonen, 2006). Microsatellites make multilocus data much easier to obtain, and their analysis is relatively inexpensive because they require little specialized equipment (Selkoe and Toonen, 2006) as outsourced analyzers are widely available.

Mosquito Microsatellites

Studies using microsatellites have determined the genetic structure of *Cx. tarsalis* populations (Barker *et al.,* 2009; Venkatesan *et al.,* 2007a*,* Venkatesan *et al.,* 2010). Fifty-seven novel microsatellite loci from *Cx. tarsalis* have been characterized (Venkatesan *et al.,* 2007b; Rasgon *et al.,* 2006). Venkatesan *et al*. (2007a) used five of these loci and a fragment of the mitochondrial reduced form of the nicotinamide adenine dinucleotide dehydrogenase 4 (*ND4)* gene to analyze the genetic structure of 12 *Cx. tarsalis* populations from California, Washington, Colorado, New Mexico, and Nebraska. The microsatellite data showed a hypothesized isolation by distance pattern and moderate genetic

structure, while the mitochondrial data showed panmixia across the states (Venkatesan *et al.,* 2007b). The microsatellite results suggest gene flow is relatively limited between populations while the mitochondrial data suggest high gene flow resulting in little isolation of the populations. Venkatesan *et al*., (2007b) suggest that this type of pattern is indicative of a past range expansion. It is difficult to differentiate the occurrence of range expansion versus panmixia because both show very similar population signatures (Venkatesan *et al.,* 2007b). The Venkatesan *et al.,* (2007b) data suggest that an initial estimation of gene flow by the mitochondrial data is exaggerated due to a past range expansion of *Cx. tarsalis*.

Gimnig *et al.* (1999) found that *Cx. tarsalis* populations in California were genetically stable over time, and temporal variation was insignificant and likely due to slight changes in population size and immigration of genetically distinct individuals over time. The Gimnig *et al.* (1999) study showed that geographic barriers, such as the Sierra Nevada and Tehachapi Mountains, acted as barriers to dispersal.

Barker *et al.* (2009) also found that geographic barriers, such as the Continental Divide in Colorado serve as a barrier to dispersal and that major waterways in the Great Plains serve as dispersal corridors for *Cx. tarsalis*. The *Cx. tarsalis* populations along the western slope of the divide in Delta County were all similar to each other yet significantly distinct from the riparian transect of northeastern Colorado (Barker *et al*., 2009).

Recent studies by Venkatesan *et al.* (2010) have shown that female mosquitoes may travel multiple kilometers to find hunting and oviposition sites. Though variation among populations of *Cx. tarsalis* in Montana is currently unknown, genetic data, in addition to behavioral data, revealed relatively high levels of migration of *Cx*. *tarsalis* in near by states (i.e., within the western US (Venkatesan *et al.,* 2010 a). This leads to the idea that *Cx. tarsalis* may be instrumental in the introduction of WNV to new reservoir populations (Venkatesan *et al*., 2010a). Further studies are needed to evaluate the importance that female *Cx. tarsalis* mosquitoes play in the spatial and temporal emergence of West Nile Virus across the breeding season.

West Nile Virus Project Goal

A multi-year study of WNV in Montana is underway at Carroll College. The overarching goal of the West Nile study is to establish a risk assessment map of the disease for the state using GIS to combine spatiotemporal layers such as vector population structure, temperature, climate, elevation, viral variation, reservoir (bird) presence, wind speed, and land cover type. This map will help assess the levels of risk based on geographical occurrence of West Nile Virus.

Microsatellite Component Project Goals

The principal objective of the present project is to investigate the spatiotemporal variation among populations of *Cx. tarsalis* across Montana to

determine the level of gene flow and migratory patterns among populations using microsatellite analysis. The hypothesis of this study is that populations separated by greater geographic distance will have higher F_{ST} values, indicating greater genetic differences between them, than those located closer together.

Methods

Mosquitoes were collected throughout the summers of 2009 and 2010 using CO_2-baited CDC light traps (Table 1).

Table 1. Collection sites including date collected, location in Montana, county, and number of individuals used in analyses.

Date Collected	Location	County	N
7/23/2009	Medicine Lake	Sheridan	10
7/15/2009	Ninepipe	Lake	14
6/16/2009	Ninepipe	Lake	10
7/14/2009	Harlem	Blaine	25
7/23/2009	Bowdoin (Site 2)	Phillips	12

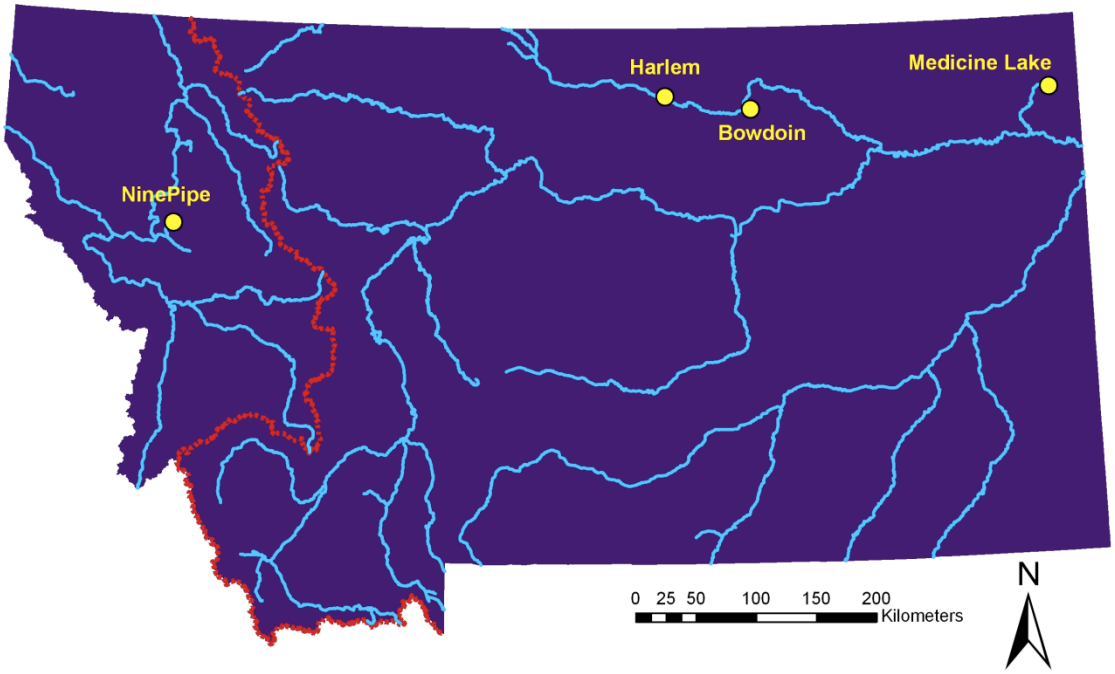

Figure 1. Map of sites analyzed.

Samples were transported on ice and stored in a -20°C freezer at Carroll College. Mosquitoes were then sorted by species. DNA was extracted from 10-25 mosquitoes from single collections at the Harlem, Bowdoin, and Medicine Lake sites (Fig. 1 and Table 1) according to the Black and DuTeau (2001) protocol. Two collections, differing in collection date by one month, were analyzed from the Ninepipe site. Individual *Cx. tarsalis* mosquitoes were placed in 1.5 mL tubes and homogenized with 25 µL of grinding buffer (0.1MNaCl. 0.2 M sucrose, 0.1 M Tris-HCl pH 9.1, 0.05 M EDTA, 0.05% SDS). This homogenate was incubated at 65°C for 30 minutes. Seven microliters (µL) of 8M KAc were added to each sample. The samples were incubated on ice for 30 minutes to precipitate the SDS, microcentrifuged for 15 minutes at 17,000 g, and the supernatant was

transferred to a new 1.5 mL test tube. Next, 100 µL of 100% ethanol were added, and the samples were incubated at room temperature to precipitate the nucleic acids. Samples were then centrifuged, the supernatant was discarded, and the remaining DNA was resuspended in nuclease-free water after being thoroughly dried by use of a Speed Vac for approximately 30-45 minutes.

PCR amplification of microsatellite products was achieved in two ways; by multiplexing five loci (CUTC6, CUTC12, CUTD107, CUTD113, CUTD120) (Rasgon *et al.*, 2006) (Table 2) and by amplifying each individual locus and pooling the five PCR products. Multiplex PCR amplification was carried out using the Qiagen Multiplex PCR Kit (2/2008, Cat. No. 206143). Primer mixes were made using 400 µL of nuclease-free water, 10 µL of each 100 µM reverse primer, 5 µL of each fluorescently labeled 100 µM forward primer, and 5 µL of each unlabeled 100 µM forward primer. PCR reactions were performed in 25 µL reaction volumes using 12.5 µL of multiplex mastermix, 2.5 µL of multiplex primer-mix, and 7.5 µL of nuclease-free water. The template DNA fulfilled the remaining 2.5 µl of volume to bring the reaction volume to 25 µl. The following cycling conditions were used: 15 minutes at 95°C followed by 32 cycles of 94°C (30 seconds), 58°C (90 seconds), 72°C (60 seconds). This was followed by a final extension at 60°C for 30 minutes.

The alternative method of amplification was also used, and loci were separated for the PCR reaction and then pooled at the completion of amplification. This was accomplished using a PCR reaction volume of 25 µL that

included 12.5 µL of Promega Go Taq Colorless Master Mix, 2X (Promega, part# M713C, lot#30136801, 9/2010), 2.5 µL of the 100 µM forward primer, 2.5 µL of the 100 µM reverse primer, and 5 µL of nuclease-free water. The remaining 2.5 µL consisted of template DNA, which brought the reaction volume to 25 µL. The following cycling conditions were used for CUTC6, CUTC12, CUTD113 and CUTD120: 5 minutes at 95°C followed by 35 cycles of 95°C (60 seconds), 59°C (60 seconds), 72°C (60 seconds) (Bio Rad MyCycler Thermocycler). This was followed by a final extension of 72°C for 10 minutes. The following cycling conditions were used for CUTD107: 5 minutes at 95°C followed by 35 cycles of 95°C (60 seconds), 64°C (60 seconds), 72°C (60 seconds). This was followed by a final extension of 72°C for 10 minutes (Bio Rad MyCycler Thermocycler).

Table 2. Loci, fluorescent label, primer sequences, and size range of microsatellite loci amplified (Rasgon *et al.,* 2006).

Locus	Fluorescent Label	Primer Sequence	Size Range (bp)
CUTC6	NED	F: 5' GCGTTTGTCATCTGGTGG 3' R: 5' GGGTTCGGAGCAGGAGTA 3'	216-228
CUTC12	VIC	F: 5' GTGGAGAACCCGTATTCAAC 3' R: 5' TACAATCACGACTCGCACATA 3'	184-211
CUTD107	PET	F: 5' ATGCCGACAGGGAGTTTC 3' R: 5' CAAAGGTCTCACGACAGAGC 3'	184-196
CUTD113	NED	F: 5' ATCATACCACTGCCCATAGTC 3' R: 5' AACCAGCAGGGACAAGTC 3'	167-176
CUTD120	6FAM	F: 5' TACCCTCGCAAACAAAACAA 3' R: 5' GTCGGCTTCCATTCCACTAC	171-180

PCR products were verified using gel electrophoresis of a 1% agarose gel with 2.5 µL of ethidium bromide (0.625 mg/ml) per 50 mL. When the product

was successfully amplified, 10 µL of the resulting sample were pooled with other PCR products for the same individual when using the second PCR method. All samples were then treated with 5 µL of ExoSap-It (USB Corporation, product code 78200/78201/78202) to eliminate excess primer and leftover DNA in the samples. The samples were then subjected to the following cycling conditions according to the ExoSap-It protocol: 37°C for 15 minutes followed by 80°C for 15 minutes (Bio Rad MyCycler Thermocycler).

Samples were sent to GeneWiz, Inc. (South Plainfield, NJ) for genotyping analysis. Samples were analyzed using a GS500LIZ_3730 standard on an Applied Biosystems 3730xl Genetic Analyzer. Applied Biosystems Peak Scanner (1.0) freeware was used to score alleles.

Statistical analyses were accomplished using FreeNA and FSTAT 2.9.3 freeware programs. Descriptive statistics including the number of alleles, heterozygosity, and allelic richness were obtained for each population. Also, F_{ST} values were used to compare genetic differentiation among populations. FreeNA provided the null allele frequencies, the F_{ST} values based on the null allele frequencies, and the genetic distance values. A null allele is an allele that fails to amplify and makes the result skewed towards a higher than actual homozygosity level in the population. The FSTAT program provided statistics about Hardy-Weinberg equilibrium values for each population.

Results

Bowdoin (site 2) had the greatest number of total alleles (40), while Medicine Lake had the highest heterozygosity (0.73), and Harlem had the greatest average allelic richness at (3.58; Table3).

Table 3. Descriptive statistics for the five populations studies (N= number of individuals sampled, H_E =heterozygosity and AR = allelic richness).

Date Collected	Location	County	N	Alleles	H_E	Average AR
7/23/2009	Medicine Lake	Sheridan	10	16	0.7256	2.8658
7/15/2009	Ninepipe	Lake	14	25	0.0016	3.2658
6/16/2009	Ninepipe	Lake	10	18	0.0007	3.0194
7/14/2009	Harlem	Blaine	25	31	0.0329	3.5852
7/23/2009	Bowdoin Site 2	Phillips	12	40	0.4163	2.9654

All 5 sites had loci that deviated significantly from Hardy-Weinberg equilibrium. Medicine Lake, Ninepipe (7/15/2009) and Ninepipe (6/16/2009) had 3 of 5 loci that deviated significantly from HW equilibrium. Bowdoin and Harlem each had 2 loci that deviated significantly from HW equilibrium. The CUTC6 and CUTD107 loci showed the greatest significant deviation from Hardy-Weinberg equilibrium due to a high proportion of homozygotes.

The CUTD120 and CUTC12 loci had the greatest null allele frequencies when compared to any of the other loci (Table 4). CUTC6 and CUTD107 had smaller null allele frequencies than any of the other loci.

Table 4. Frequency of null alleles by locus and population.

		Null allele Frequencies			
Population			Locus		
	CUTD120	CUTC12	CUTD113	CUTC6	CUTD107
Medicine Lake	0	0.00001	0	0.00001	0
Ninepipe 7/15/2009	0.14189	0.14309	0	0.00001	0.00001
Ninepipe 2 6/16/2009	0.35883	0.15842	0	0	0.00002
Harlem	0.16425	0	0.0671	0.00001	0
Bowdoin Site 2	0	0.0421	0.00001	0.00001	0.00001

The F_{ST} statistic gives valuable insight into the genetic variation and thus evolutionary relationship within and among natural populations. The F_{ST} value can range from 0 to 1. A value of 0 means that panmixia is occurring and the two study populations are very closely related due to a high rate of gene flow. A value of 1 means that the populations are genetically isolated from one another and no gene flow occurs between them. The data indicate (Table 5) that the Medicine Lake (Sheridan County) population is significantly different from the Harlem (Blaine county) population ($F_{ST} = 0.034$, $p = 0.042$). The Ninepipe population on 7/15/2009 is significantly different from Harlem ($F_{ST} = 0.048$, $p = 0.049$). The Ninepipe 6/16/2009 population is nearly significantly different from Harlem ($F_{ST} = 0.044$, $p = 0.055$). Medicine Lake is significantly different from the Bowdoin population ($F_{ST} = 0.044$, $p = 0.017$). All other pairwise comparisons (6/10) indicate populations do not significantly differ in F_{ST} values (Table 5).

Table 5. A pairwise population comparison of geographic distance (upper diagonal) and F_{ST} value (lower diagonal).

		Geographic Distance (km)			
	Medicine Lake	Ninepipe 7/15/2009	Ninepipe 6/16/2009	Harlem	Bowdoin
Medicine Lake	-	740	740	320	250
Ninepipe 7/15/2009	0.011	-	0	420	490
Ninepipe 6/16/2009	0.042	-0.014	-	420	490
Harlem	0.033**	0.048**	0.044**	-	72
Bowdoin	0.044*	0.039	0.031	0.012	-
		F_{ST}			

Note: **p < 0.05, *p <0.06

Discussion

The primary objective of this study was to determine if a relationship exists between F_{ST} values and geographic distance in the *Culex tarsalis* populations of Montana. I hypothesized that populations separated by a greater geographic distance would have higher F_{ST} values than those located closer together.

These data do not consistently support the hypothesis that F_{ST} values increase as geographic distance increases. If my hypothesis were supported, Medicine Lake and Bowdoin would have a smaller F_{ST} value than Harlem and Medicine Lake, which are separated by a larger geographic distance along the Milk and Missouri Rivers. This is not the case, though, because Harlem and Medicine Lake have a

lower F_{ST} than that of Medicine Lake and Bowdoin (Table 5). Similar deviations from predicted patterns based on previous studies (Barker *et al*., 2009; Venkatesen *et al*., 2007b) are seen in many pairwise comparisons between Montana populations.

Some population comparisons do seemingly support a hypothesis of increasing genetic differentiation between populations that are separated by greater distances. For example, the two Ninepipe populations, collected on different dates, show the least differentiation in population structure. However, the lowest F_{ST} value acquired was between Ninepipe 7/15/2009 and Medicine Lake, which are separated by the greatest geographic distance of all population pairs.

The data may not be presenting the patterns found by other similar studies (Barker *et al*., 2009; Venkatesen *et al*., 2007b) due to the extremely low sample sizes in this study. Alleles were not scored for every individual at every locus due to excessive contamination and amplification inconsistencies during the summer of 2010. The small sample sizes could be the cause of the high frequency of detected null alleles at certain loci (Table 4). Additionally, deviations from Hardy-Weinberg Equilibrium (HWE) indicate that observed heterozygosity levels are lower than expected, indicating an excess in the number of observed homozygotes. These three factors – small sample sizes, high frequencies of null alleles, and deviations from HWE – are almost certainly confounding the data and may or may not explain why the hypothesized

patterns are not recognized. Nonetheless, this preliminary study contributes to the protocol development of microsatellite studies of *Cx. tarsalis*. A more efficient protocol will continue to be developed in subsequent summers.

A pattern of increased genetic isolation by geographic distance for *Cx. tarsalis* populations is expected based on patterns observed for *Cx. tarsalis* in other states with similar dispersal corridors and barriers. Venkatesan *et al*., (2007b) found an isolation by distance pattern for microsatellite and mitochondrial data of mosquitoes. That is, the populations became more distinct from one another as the geographic distance increased. Gimnig *et al.* (1999) found significant differences in the genetic structure of populations separated by large geographic distance and geographic barriers such as the Sierra Nevada and Tehschapi Mountains. Barker *et al.* (2009) found that those populations connected by the waterways of the Great Plains became increasingly different with distance, and that those on opposite sides of the continental divide were genetically distinct, suggesting that genetic structure is related to geographic features.

The hypothesized pattern of genetic isolation by distance was not observed in the present study with the limited data that could be analyzed. The previously mentioned problems – low sample size, high null allele frequencies, and deviations from HWE - were too great to be able to draw many conclusions from the data. In some pairwise comparisons, the hypothesized pattern was observed, but it was very inconsistent, and other comparisons showed the

opposite of what was hypothesized. Further studies that include more individuals per site as well as more sites are necessary to discern the patterns of genetic structure of *Cx. tarsalis* in Montana.

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