Microsatellite Analysis of Gene Flow between Culex tarsalis Populations Connected by Irrigation in Yellowstone County, Montana

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Microsatellite Analysis of Gene Flow between *Culex tarsalis* Populations Connected by Irrigation in Yellowstone County, Montana

Submitted in partial fulfillment of the requirements for graduation with honors from the Department of Natural Sciences at Carroll College, Helena, Montana

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April 2013
This thesis for honors recognition has been approved for the Department of Natural Sciences by:

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Acknowledgements

I would like to thank Dr. Jennifer Geiger for all of her support and guidance and for the opportunity to understand the true meaning of “research.” Her encouragement and persistence has fueled the countless hours I have spent in the lab. I thank Dr. Grant Hokit for going out into the field to gather the samples for this thesis as well as providing a map of their locations. I also thank him for his assistance in helping me calculate and analyze the results. I thank Dr. Gerald Shields for his critiques for the initial writing stage of this thesis. I thank MT-INBRE for their financial backing. This research was supported by a grant from the National Center for Research Resources (P20 RR16455-09), a component of the National Institutes of Health. I thank Kellie O’Rourke for her mentoring and continued inspiration after her graduation. I thank Keeli Nelson for her assistance in gathering data this past summer. I thank Paige Esposito and Max Bernt for their support and camaraderie for the past two years. I thank Carroll College for providing research lab facilities. Finally, I thank my friends and family for their continued encouragement and optimism.
Table of Contents

Acknowledgments-----------------------------------------------3
Abstract--------------------------------------------------------5
List of Tables-----------------------------------------------6
List of Figures-----------------------------------------------7
Introduction-----------------------------------------------8
Materials and Methods---------------------------------------11
Results-----------------------------------------------14
Discussion-----------------------------------------------17
Literature Cited-----------------------------------------------19
Abstract

The purpose of this study was to determine whether population connectivity by irrigation enhances gene flow between *Culex tarsalis* populations in the state of Montana. Four populations of *Cx. tarsalis* were collected along the Yellowstone and Bighorn rivers and allele frequencies obtained from PCR amplification of four microsatellite loci, visualized using the QIAxcel Advanced System, were used to assess the genetic structure of the populations. Results of the four loci indicate lower pairwise $F_{ST}$ values between only two of three populations connected through waterway, which suggests a disparity in the data. If the genetic similarity between these populations reflects rates of gene flow, these results suggest that higher degrees of gene flow may not be due to irrigation but rather to connectivity by any waterway. Further analysis of additional polymorphic microsatellite loci needs to be performed to determine if a higher degree of gene flow does indeed occur between populations connected by irrigation.
List of Tables

Table 1. Allele Size per Locus-----------------------------------------------14
Table 2. Allele Size Most Frequently Observed-----------------------------15
Table 3. Unique Allele Sizes-----------------------------------------------15
Table 4. Null Allele Frequencies------------------------------------------16
Table 5. Pairwise Population $F_{ST}$ Values-------------------------------16
List of Figures

Figure 1. Sampling locations in Yellowstone County---------------------------------12
Introduction

Background

West Nile Virus (WNV) (*Flaviviridae: Flavivirus*) is an arbovirus that was first detected in Uganda in 1937 (Clements 2012; Smithburn *et al.* 1940). The first case of WNV in the United States was discovered in 1999 in New York (CDC 1999) and by 2002 it was detected in Montana (Huhn *et al.* 2003). The strain of West Nile Virus detected in New York in 1999 was determined to be most similar to the Israeli strain (Lanciotti *et al.* 1999), and it is believed that WNV was brought to the United States through the import of exotic birds because two zoos in New York had infected exotic birds in the summer of 1999 (Clements 2012). The main vectors of WNV are mosquitoes, however not all mosquitoes are competent vectors of the virus. For a mosquito to be a competent vector of WNV it must come into contact with the virus while taking a bloodmeal. Once the mosquito ingests the virus it needs to pass through the midgut epithelium cells, replicate, and enter into the salivary glands (Clements 2012). From there the virus must eventually make its way into the salivary ducts where it can then infect its next host by being released while the mosquito takes a bloodmeal (Clements 2012).

Most people that are infected with WNV are asymptomatic, while some experience symptoms much like the flu. Symptoms, however, can be as severe as inflammation of the brain and nerve cells along with cell death and localized bleeding (Marra *et al.* 2004). WNV infection outcomes can be proportioned to: 100 asymptomatic: 30 febrile: 1 neuroinvasive (Clements 2012). Since 1999 there have been a total of 36,801 cases of WNV in the United States with 1,506 of those cases leading to death. Montana has seen 508 cases of WNV with only nine fatalities since its introduction to the state (CDC 2012). Because the majority of people are asymptomatic, however, these infection numbers could actually be much higher.

WNV epidemics arise when wet springs are followed by hot, dry summers (Marra *et al.* 2004). Likewise, higher temperatures shorten the extrinsic incubation period (EIP), which is the time after receiving an infectious bloodmeal that females become infected and are able to transmit the virus (Reisen *et al.* 2006). Thus early onset peak temperatures
lead to increased amplification of the virus and subsequently increased human incidence rates (Reisen et al. 2009). Culex tarsalis mosquitoes, the primary vector in the western US, are an enzootic vector, infecting avian amplifying hosts, as well as a bridge vector, meaning they can transmit the virus from an amplifying host to a dead-end host (Clements 2012). In the early summer season Kent et al. (2009) found that Cx. tarsalis primarily feed on the American Robin (Turdus migratorius) and Mourning Doves (Zenaida macroura) and shift to feeding on mammals near the end of the season due to a lack of preferred avian hosts. This behavior may be the explanation for greater human rates of WNV in the latter part of the season. The speed with which WNV spread across the United States caused scientists to believe that migratory birds were responsible for the movement (Rappole et al. 2000), however for the 2000 and 2001 outbreaks Rappole and Hubalek (2003) indicated that the pattern of WNV movement did not show long-distance “leap-frog” motions, but rather radiated outward from all directions. Due to this information, more recent suggestions have indicated that the virus spread more through the random movement of resident birds (Reisen et al. 2004). Venkatesan and Rasgon (2010), however, found evidence of high gene flow across large geographic distances for Cx. tarsalis, which in combination with Cx. tarsalis’ ability to travel up to four km in a day (Reisen and Reeves 1990), may suggest a role for Cx. tarsalis in the dispersal of the virus across the United States.

Cx. tarsalis populations are typically more abundant at elevations between 1200 and 1450 m (Barker et al. 2009). This species also prefers rural habitats (Epstein and Defilippo 2001), and breeds in newly created standing water that is high in organic material (Beehler and Mulla 1995). River corridors near irrigated lands, which may be effective breeding grounds for Cx. tarsalis, are now being considered as a means for dispersing these mosquitoes into nearby populations (Eisen et al. 2010). These findings have led to more studies looking at the importance of irrigation to the development of WNV. The same study by Eisen et al. (2010) showed that there is a meaningful positive affiliation between shorter distance to irrigated agriculture and elevated WNV prevalence in Colorado where the landscape is similar to Montana. Likewise, positive correlations between rural agricultural settings and WNV incidence were found in Iowa (DeGroote et al. 2008), California (Reisen et al. 1992), and Texas (Cardenas et al. 2011). From 2002 to
2007, Montana saw a jump from 1.967 million acres of irrigated land to 2.013 million acres (USDA 2007). The majority of Montana inhabitants reside in rural communities, many of which center around the agricultural industry. This landscape provides ideal habitat for *Cx. tarsalis* development as well as bird hosts (Cardenas *et al.* 2011). Because of this, risk levels should be higher near irrigation due to the number of females feeding on infected birds and later on dead-end hosts. This study utilizes microsatellites to determine gene flow of *Cx. tarsalis* and WNV through irrigated land.

**The Utility of Microsatellites**

Microsatellites are tandem repeats of one to six nucleotides that have been useful determinants in other studies to estimate migration and individual relatedness (Selkoe and Toonen 2006). The number of alleles per locus can indicate the diversity of a population (Allendorf 1986), which can be valuable information when determining if gene flow occurs between two distinct regions. Rasgon *et al.* (2006) characterized six polymorphic microsatellite loci for *Cx. tarsalis* that were in Hardy-Weinberg equilibrium. Three of these loci were used in this study to determine genetic diversity between different populations in the Yellowstone County with the addition of one locus from Venkatesan *et al.* (2007). The loci used were: CutC6, CutD120, CutD107, and CutC201. Microsatellites have yet to be used to determine gene flow between populations of *Cx. tarsalis* in areas of irrigated land. Previous investigations analyzed land cover data, human incidence rates, and prevalence of *Cx. tarsalis* near areas of agricultural activity. However, in terms of measurement of gene flow, previous studies have utilized microsatellite markers to calculate $F_{ST}$ values which can provide a measurement of population divergence (Neigel 2002).

**The West Nile Virus Project at Carroll College**

The goal of the West Nile Virus project at Carroll College is to produce a risk assessment model for the state of Montana. To develop this model geographic information systems and molecular characterization and detection methods are being used. The model will include information such as elevation, temperature, bird hosts,
precipitation, vegetation, virus and vector frequency, and vector genetic diversity (Hokit et al. 2008).

The specific aim of this study is to more deeply examine gene flow between Cx. tarsalis populations to see if irrigation enhances gene flow and thus exchange of WNV. My hypothesis is that populations connected by irrigation systems will be more genetically similar than those that are disconnected. Likewise, populations that are connected through a nearby river but not connected through irrigation will be more genetically alike than those disconnected through both irrigation and nearby waterways. There are already known barriers to gene flow such as mountain ranges, arid conditions, and large geographic distances (Gimnig et al. 1999), however, enhancers to gene flow are relatively unknown with waterway connectivity and short geographic distances among the few actually studied. Based upon previous research, however, irrigation could be a new measure of enhanced gene flow.

**Materials and Methods**

Mosquito samples were collected using CDC CO₂ baited traps from four fishing access sites along the Yellowstone and Bighorn rivers: Gritty Stone (Yellowstone River), Voyagers Rest (Yellowstone River), Bundy Bridge (Yellowstone River) and Arapooish (Bighorn River). The Voyagers Rest and Bundy Bridge locations are not only connected by waterway, but are also connected by the same flood irrigation zone. Gritty Stone is connected to these two sample sites by the same waterway, however, it is not connected by irrigation, and Arapooish is not connected to any of these sample sites by either waterway or irrigation (Figure 1). Collected mosquitoes were stored at -20° C overnight and sorted based upon identification. A minimum of 30 Cx. tarsalis mosquitoes were required for a population.
The DNA extraction protocol described by Black and DuTeau (1997) was performed on mosquitoes. Individual mosquitoes were placed in 1.5 mL round-bottom tubes with 25 μl of grinding buffer (0.1 M NaCl, 0.2 M Sucrose, 0.1 M Tris-HCl pH 9.1, 0.05 M EDTA, 0.05% SDS) and ground with glass pestles until insect parts could not be seen. The pestle was then washed with an additional 25 μl of grinding buffer to remove any residual DNA. The contaminated pestle was rinsed with water and HCl before repeated use. Mosquitoes were briefly centrifuged to get the homogenate down to the bottom of the tube before incubating it at 65°C for 30 minutes. Immediately following incubation, 7 μl of 8 M potassium acetate (KAc) was added to each tube and placed on ice for another 30 minute incubation. The tubes were then centrifuged at 15,000 g for 15 minutes followed by transfer of the supernatant to a new 1.5 mL tube. One hundred microliters of 100% ethanol kept in a -20°C freezer was then added to each tube before a 5 minute incubation at room temperature. Tubes were centrifuged at 15,000 g for 15 minutes, the ethanol was removed, and an additional 100 μl of 100% ethanol was added.
before centrifugation at 15,000 g for 5 minutes. The ethanol was removed again and the tubes were placed in the Speed Vac to dry. The pellets were then resuspended in 200 μl of TE (0.01 M Tris-HCl pH 8.0, 1 mM EDTA) and vortexed to break up the pellet before being stored at -20°C.

The microsatellite loci used were CutC6 (Rasgon et al. 2006), CutD120 (Rasgon et al. 2006), CutD107 (Rasgon et al. 2006) and CutC201 (Venkatesan et al. 2007). PCR amplification of these microsatellite loci was conducted following the procedure of Venkatesan et al., (2007). PCR was set up in 25 μl reactions containing 0.8 units of Taq polymerase, 2.5 μl of 10X Thermopol buffer (New England Biolabs), 0.2 mM of each dNTP, 10 μM of each microsatellite primer, 16.6 μl of PCR certified water, and 2.5 μl of sample DNA. All microsatellite loci were run with the following cycling conditions: 95°C for 5 min; 10 cycles of 94°C for 30 s, 57°C for 1 min, and 72°C for 30 s; 27 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s; followed by a 10 minute extension at 72°C (Bio Rad MyCycler Thermocycler).

Gel electrophoresis was used to confirm amplification with 1% agarose gels loaded with 4 μl of each sample and stained with 2.5 μl of ethidium bromide (0.625 mg/mL) per 50 mL.

The QIAxcel Advanced System was then used to determine the size of alleles present at each microsatellite locus. The QIAxcel Advanced User Manual, DNA High Resolution Kit and Cartridge, QX 25-500 bp Size Marker diluted to 30 ng/μl and the QX 15 bp-600 bp Alignment Marker were used in conjunction with the QIAxcel Advanced System to base call alleles (QIAGEN 2011). Base pair peaks were considered valid if they have a minimum concentration of 2.61 ng/μl. Homozygotes were defined as individuals with one peak that has a concentration of at least double the next highest peak’s concentration. Samples that had two peaks were considered heterozygotes if the lower peak was at least half the concentration of the higher peak. Only individuals with valid peaks in the region of 140 to 260 base pairs were used as data. Any samples with more than two peaks of similar concentrations were considered missing data, as well as any samples with the highest concentration peaks outside of the base pair range (Geiger
pers. comm.). FreeNA was used to analyze relatedness of individuals and thus estimate the level of gene flow between populations. This software estimates null allele frequencies for microsatellite datasets and calculates $F_{ST}$ values that have been corrected for the presence of high null allele frequencies (Chapuis and Estoup 2007). Null alleles are those in which a mutation has occurred which either prevents PCR amplification completely or amplifies only one homologue which can lead to an overestimate of homozygotes (Callen et al. 1993). $F_{ST}$ values can be defined based upon the inbreeding coefficient of a population or the differences in allele frequencies (Neigel 2002).

**Results**

Loci amplification did occur within the 160 to 260 base pair range. Amplification of CutC6, CutD120, and CutD107 revealed nine alleles while CutC201 contained eleven alleles (Table 1).

<table>
<thead>
<tr>
<th>CutC6 (9)</th>
<th>CutD120 (9)</th>
<th>CutD107 (9)</th>
<th>CutC201 (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>186</td>
<td>165</td>
<td>166</td>
<td>204</td>
</tr>
<tr>
<td>198</td>
<td>168</td>
<td>184</td>
<td>207</td>
</tr>
<tr>
<td>213</td>
<td>171</td>
<td>190</td>
<td>210</td>
</tr>
<tr>
<td>216</td>
<td>174</td>
<td>193</td>
<td>213</td>
</tr>
<tr>
<td>219</td>
<td>177</td>
<td>196</td>
<td>216</td>
</tr>
<tr>
<td>222</td>
<td>180</td>
<td>199</td>
<td>225</td>
</tr>
<tr>
<td>228</td>
<td>183</td>
<td>205</td>
<td>228</td>
</tr>
<tr>
<td>231</td>
<td>189</td>
<td>211</td>
<td>231</td>
</tr>
<tr>
<td>288</td>
<td>237</td>
<td>217</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>237</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>240</td>
</tr>
</tbody>
</table>

The Gritty Stone, Voyagers Rest and Bundy Bridge populations all shared the same most frequent allele size for locus 6, 107 and 201 (Table 2). All populations sampled contained at least one allele in common for each locus and four CutD201 alleles were common to all populations (data not shown). All of the populations with the
exception of Arapooish had at least one allele that was unique for a particular locus (Table 3).

Table 2: The allele size most frequently observed for each population broken down by locus. VR=Voyagers Rest, GS=Gritty Stone, BB=Bundy Bridge, AP=Arapooish

<table>
<thead>
<tr>
<th>Loci</th>
<th>Allele size most frequent in VR</th>
<th>Allele size most frequent in GS</th>
<th>Allele size most frequent in BB</th>
<th>Allele size most frequent in AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CutC6</td>
<td>216</td>
<td>216</td>
<td>216</td>
<td>219</td>
</tr>
<tr>
<td>CutD120</td>
<td>180</td>
<td>177</td>
<td>174</td>
<td>174</td>
</tr>
<tr>
<td>CutD107</td>
<td>193</td>
<td>193</td>
<td>193</td>
<td>193</td>
</tr>
<tr>
<td>CutC201</td>
<td>228</td>
<td>228</td>
<td>228 and 231</td>
<td>207</td>
</tr>
</tbody>
</table>

Table 3: Allele sizes that are unique to each sample site and number of times present within population.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Voyagers Rest</th>
<th>Gritty Stone</th>
<th>Bundy Bridge</th>
<th>Arapooish</th>
</tr>
</thead>
<tbody>
<tr>
<td>CutC6</td>
<td>186 (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CutD120</td>
<td>-</td>
<td>189 (2), 237 (2)</td>
<td>183 (2)</td>
<td>-</td>
</tr>
<tr>
<td>CutD107</td>
<td>217 (1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CutC201</td>
<td>-</td>
<td>240 (2)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Analysis Using FreeNA:

The FreeNA program was run for all sample sites at locus 6, 120, 107 and 201. Any individual samples that did not amplify at two of the four loci were removed from the data set before the analysis was performed. The null allele frequencies indicated that only the Voyagers Rest population at locus 107 was below a normal estimate for null allele frequency (Table 4). Populations were considered to have a high null allele frequency if the value was 20% or above.
Table 4: Null allele frequencies for each population at locus 6, 120, 107 and 201. The bolded value indicates the site that had a low null allele frequency. VR=Voyagers Rest, GS=Gritty Stone, BB=Bundy Bridge, AP=Arapooish

<table>
<thead>
<tr>
<th>Locus</th>
<th>Population</th>
<th>Null Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>VR</td>
<td>0.30941</td>
</tr>
<tr>
<td>6</td>
<td>GS</td>
<td>0.26563</td>
</tr>
<tr>
<td>6</td>
<td>BB</td>
<td>0.26122</td>
</tr>
<tr>
<td>6</td>
<td>AP</td>
<td>0.36676</td>
</tr>
<tr>
<td>120</td>
<td>VR</td>
<td>0.38023</td>
</tr>
<tr>
<td>120</td>
<td>GS</td>
<td>0.40501</td>
</tr>
<tr>
<td>120</td>
<td>BB</td>
<td>0.39631</td>
</tr>
<tr>
<td>120</td>
<td>AP</td>
<td>0.41120</td>
</tr>
<tr>
<td>107</td>
<td>VR</td>
<td>0.13057</td>
</tr>
<tr>
<td>107</td>
<td>GS</td>
<td>0.27595</td>
</tr>
<tr>
<td>107</td>
<td>BB</td>
<td>0.34953</td>
</tr>
<tr>
<td>107</td>
<td>AP</td>
<td>0.30909</td>
</tr>
<tr>
<td>201</td>
<td>VR</td>
<td>0.33297</td>
</tr>
<tr>
<td>201</td>
<td>GS</td>
<td>0.39387</td>
</tr>
<tr>
<td>201</td>
<td>BB</td>
<td>0.40773</td>
</tr>
<tr>
<td>201</td>
<td>AP</td>
<td>0.35867</td>
</tr>
</tbody>
</table>

FreeNA also provided an analysis of pairwise $F_{ST}$ values that accounted for high null allele frequency. Two populations that had an $F_{ST}$ value of less than 0.015 were considered to not be significantly different from each other (Hokit pers. comm.). All of the populations were distinct from each other except for the Gritty Stone and Bundy Bridge populations which were considered to be the same (Table 5).

Table 5: Pairwise population $F_{ST}$ values for all loci. The bolded value is the one pairwise comparison in which the populations are genetically indistinguishable. VR=Voyagers Rest, GS=Gritty Stone, BB=Bundy Bridge, AP=Arapooish

<table>
<thead>
<tr>
<th>Population</th>
<th>VR</th>
<th>GS</th>
<th>BB</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS</td>
<td>0.029403</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>0.030320</td>
<td>-0.000830</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>0.063319</td>
<td>0.067653</td>
<td>0.055176</td>
</tr>
</tbody>
</table>
Discussion

The objective of this study was to determine if higher degrees of pairwise gene flow existed between Cx. tarsalis populations that were connected by irrigation which could also suggest movement patterns of WNV. My hypothesis was that populations connected by irrigation systems are more genetically similar than those disconnected. Likewise, populations that are connected through a nearby river but not connected through irrigation are more genetically alike than those disconnected through both irrigation and nearby waterways.

The results of this study indicate that a higher pairwise gene flow between populations connected through irrigation does not exist and that populations geographically separated by waterway and irrigation are genetically different (Table 5). Statistical data analysis using FreeNA for loci 6, 120, 107 and 201 indicates that there are high null allele frequencies within this data set (Table 4). Null alleles have been known to cause deviations from Hardy-Weinberg Equilibrium (HWE) (Callen et al. 1993). A smaller than expected number of heterozygous individuals, as seen in this data set, can then account for populations failing to adhere to HWE.

The pairwise $F_{ST}$ values indicated that the Gritty Stone and Bundy Bridge populations could be considered genetically indistinguishable whereas all the other populations were considered distinct from one another. Populations exhibited the greatest difference when compared to the Arapooish population (Figure 1 and Table 5). This was as expected due to the large geographic distance and degree of waterway separation between Arapooish and the other three populations. However, it would be expected that Gritty Stone and Voyagers Rest would be more genetically similar because of the short geographic distance separating them as well as their connectivity by water. Likewise, Voyagers Rest and Bundy Bridge would be expected to be genetically similar due to their connectivity by waterway and flood irrigation zone. The data indicated that Gritty Stone and Bundy Bridge were genetically similar even though they only share connectivity by waterway but do not share the additional factor of close geographic proximity or connection through a flood irrigation zone. However, due to the majority of populations exhibiting genetic distinction from one another it may be that Gritty Stone and Bundy...
Bridge are indeed distinct sites. Nevertheless, it is difficult to distinguish the true relationship between these populations because all, with the exclusion of one, exhibited high null allele frequencies for each locus which could explain either the similarity between Gritty Stone and Bundy Bridge or the distinctness of the other populations. This disparity in the data obtained could additionally be due to the small population size and limited number of loci used.

As previously noted, these results are unexpected, especially upon comparison to previous studies on irrigation and WNV. For example, Cardenas et al.’s (2011) 2003 to 2010 case study indicated that a proximity to irrigation canals increased the risk of WNV for residents of El Paso County, Texas by six fold. He observed that 43% of human WNV cases reported during these seven years were within 330 meters of irrigation canals while only 8% of WNV cases lived greater than 7400 meters away (Cardenas et al. 2011). Likewise, research by Eisen et al. (2010) found that in Colorado a distance of less than one kilometer from irrigated agricultural land was a statistically significant risk factor for obtaining WNV during 2003 and 2007. This suggested that the larger the Cx. tarsalis larvae habitats, the greater the WNV incidence rate (Eisen et al. 2010). Eisen et al. (2010) also proposed that rivers near these areas of irrigated land, which are considered breeding grounds for Cx. tarsalis, could be the means of dispersing the vector into nearby populations. With these studies in mind, it would be expected that gene flow should be enhanced due to the high proportion of agricultural land in the state of Montana which is optimal Cx. tarsalis breeding ground and bird host habitat.

Barker et al.’s (2009) study additionally suggested that Cx. tarsalis disperses along waterways in Colorado. They indicated that a higher degree of pairwise gene flow existed between populations that are less than 190 kilometers apart (Barker et al. 2009). This suggests that if a higher degree of pairwise gene flow does not exist between populations connected through irrigation then the Gritty Stone, Voyagers Rest and Bundy Bridge populations connected through waterway should exhibit greater levels of gene flow, which was not found here.

The results of this study are unclear and could suggest that populations connected through waterway exhibit a greater degree of gene flow or that for this particular
sampling year populations are genetically distinct from one another. Additional studies should include multiple locations connected through a variety of irrigated areas over numerous WNV seasons as well as the use of additional microsatellite loci. This study does not indicate that a higher degree of gene flow exists between populations connected through irrigation. As previously mentioned, high null allele frequencies and small population size could be reason for this result.

**Literature Cited**


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