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Microsatellite Analysis of *Culex tarsalis* Populations in Montana

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Abstract

West Nile Virus (WNV) has been present in Montana since 2002. The primary vector of WNV in Montana is *Culex tarsalis.* At Carroll College, the purpose of the WNV project is to survey for the presence of the virus across the state and create a risk model in real time. The goal of this particular study was to find a microsatellite protocol that consistently works in order to create a reliable landscape genetics model for *C. tarsalis* populations. Three reagent sets were used in order to test which reagent conditions best amplified the three loci, followed by gel electrophoresis to visualize the amplification. The results indicated degradation of the DNA, suggesting new DNA is needed to test which conditions produced the best amplification.

Introduction

Background

In the United States, West Nile Virus (WNV) was first detected in New York City in 1999 (Sejvar, 2003). From there, it began to spread, first to other areas of New York before spreading across the United States. By 2002, WNV had reached the West Coast region (Sejvar, 2003). It was detected in Montana in 2002; a horse was first diagnosed then two neuroinvasive cases were detected in humans (CDC, 2002). Blood serum tests showed that WNV is capable of infecting birds and mammals, particularly horses and humans (Sejvar, 2003). West Nile Virus has been detected in humans in Montana every year since 2002, with the exception of 2010 (CDC, 2018).

West Nile Virus belongs to the family *Flaviviridae*, which also includes Zika virus, yellow fever virus, and dengue virus. WNV is an arbovirus, meaning it is an arthropod-borne virus (CDC, 2003). Such viruses cycle between arthropod (in this case, mosquitoes) and vertebrate hosts (namely birds, horses, and humans). Mosquitoes acquire the virus by biting an infected bird, which serves as a reservoir for WNV (Hayes et al., 2005). In the bird host, the virus replicates and persists in the blood system without causing disease. In an infected mosquito, WNV replicates in the salivary glands, and the mosquito transmits the virus to others during blood meals (CDC, 2017). The mosquito is only able to infect others once the virus has reached the salivary glands.

Most people (80%) infected with WNV show no symptoms. The majority of the remaining people infected show flu like symptoms such as vomiting, diarrhea, body aches, and fever (Hayes et al., 2005). Rarely, people experience neuroinvasive symptoms, including inflammation of the brain or meningitis. Elderly people are at greater risk for

developing these symptoms due to weaker immune systems; however, these symptoms appear in about one in 150 cases of WNV. Of those diagnosed with this form, one in ten people die from the disease (CDC, 2017).

West Nile Virus can be found in various mosquito species, with *Culex* being the most common genus for carrying WNV (Hayes et al., 2005). The most common vector species in Montana are *Culex tarsalis* and *Culex pipiens*, with *C. tarsalis* accounting for most of the cases (Goddard et al., 2002). *Culex tarsalis* is the predominant vector of WNV west of the Mississippi River. The virus may be able to survive colder temperatures in this species, which is necessary in Montana (Hayes et al., 2005).

West Nile Virus Research at Carroll College

Carroll College has been involved with West Nile Virus surveillance since 2009. Partnerships with Chief Dull Knife College, Aaniiih Nakoda College, and Montana State University exist to trap and identify mosquitoes by species. Carroll College also partners with the State of Montana Public Health Laboratory to test the vector species for WNV. The purpose of this program is to have real time reporting of WNV in Montana and to generate a risk model of WNV in Montana (Alvey, 2014).

Use of Microsatellites

The presence of WNV in Montana varies depending on location. Typically, WNV is more common in eastern Montana than in western Montana (Hokit et al., 2013). This evidence suggests barriers to *C. tarsalis* gene flow between the eastern and western parts of the state. Landscape genetics can be a useful tool in determining the degree of interactions between the populations of *C. tarsalis* in Montana that may lead to the spread

of WNV because there are many geographical features that could influence gene flow and genetic variation between populations in different parts of the state.

The use of microsatellites is helpful for creating reliable genetic population maps of *C. tarsalis* as seen in previous studies around the Western United States (i.e., Rasgon et al., 2006; Venkatesen et al., 2007; Barker et al., 2009). Microsatellites are a form of tandem repeats. Typically, microsatellites are one to ten nucleotides long, with most being less than six nucleotides in length. Because tandem repeats have high mutation rates, they are useful tools in genetic analysis of populations. They provide more resolution than inter-simple sequence repeats (ISSRs) and mitochondrial DNA (mtDNA)—two other commonly use molecular markers (Vieira et al., 2016). The number of alleles per locus can determine how closely related different populations of a species are (Vieira et al., 2016). The first study to create a molecular marker suitable for investigating the population structure of *C. tarsalis* utilized clone libraries (Rasgon et al., 2006). They did so by extracting DNA and creating DNA libraries of *C. tarsalis* and sequencing clones from these libraries to identify the microsatellite markers. They identified six polymorphic microsatellite loci in Hardy-Weinberg equilibrium in *C. tarsalis*, which can be used to determine gene flow between *C. tarsalis* populations in Montana.

Since WNV is seen more in eastern Montana than in western Montana, there could be a difference in the WNV transmission potential of the different populations. Other studies, including Venkatesan et al. (2007) and Kothera et al. (2009), have used these microsatellite markers to analyze *Culex* populations in different states. The results in Venkatesan et al. (2007) show that populations seem to vary with geographical

structures like mountains and waterways, which suggest that these barriers affect gene flow. In Colorado, the Continental Divide has been shown as a powerful barrier for gene transfer of *C. tarsalis* (Barker et al., 2009). These studies suggest microsatellite analysis of *C. tarsalis* populations in Montana could be useful for understanding genetic structure because, similarly, many waterways and the Continental Divide separate mosquito populations.

Though past studies have shown the usefulness of microsatellite analysis in understanding population genetic structure of *C. tarsalis*, this method has not been consistently successful at Carroll. Because the microsatellite method has been unreliable, it is difficult to create an accurate model of the gene flow among these populations. McKeown started these studies in 2011 with microsatellites, but the study yielded results that contradicted previous studies. Unlike previous studies, this study did not show that the populations connected via waterways had greater gene flow. Two possibilities listed as a cause for this result are the high frequency of null alleles and small populations sizes. A study done by Casey (2013) also yielded differing results, likely due to the same issues. Since those studies resulted in many null alleles, Fiocchi tried mtDNA in 2016, using a sequence commonly used for comparison of invertebrates (Folmer et al., 1994). This sequence analysis did not reveal any significant genetic structure. Next, Lockman (2017) tried ISSR markers because the mtDNA did not provide the resolution needed. ISSRs are highly variable markers that have been useful for elucidating population genetics patterns in many plant species (Lawson & Zhang, 2006). Lockman (2017) showed there is ISSR variation in the *C. tarsalis* populations in Montana, but contrary to

previous studies (i.e., Barker et al., 2009), did not show a differentiation between groups on either side of the Continental Divide.

Purpose of this Study

The purpose of this study was to adjust the original microsatellite protocol used in to be able to use it as an effective tool for distinguishing mosquito populations in Montana (Rasgon et al., 2006). Specifically, the goal was to optimize the microsatellite technique by varying the thermocycler conditions, reagents, and reagent concentrations. This would allow for future studies of gene flow in *C. tarsalis* populations in Montana. I hypothesized that the best conditions and reagents for PCR amplification would be those outlined in the original study identifying microsatellite markers in *C. tarsalis* (Rasgon et al., 2006).

Materials and Methods

Mosquito Collections

Mosquitoes were collected using CO_2 traps around the state of Montana between the years of 2009 and 2017. The traps were set up in the evening then collected the next morning. The collected mosquitoes were stored at -20°C then sorted into their species. *Culex tarsalis* and *Culex pipiens* were separated from non-target mosquito species. Only *C. tarsalis* were used for DNA extractions.

DNA Extraction

The mosquito DNA used for this experiment was extracted in previous years, using the protocol outlined by Black and DuTeau (2001). Each extraction contained the DNA from a single mosquito within the population of the collection site. The samples were diluted

in order to have an equal DNA concentration in each DNA sample. The samples were stored at -20 °C until use. The DNA used for this experiment was from 2012. DNA samples were chosen based on which samples had the best amplification in previous studies.

Molecular Methods

Three different reagent sets were used for this experiment. The first reagents tested included Standard Taq Reaction Buffer and Taq DNA polymerase. The second set of reagents tested replaced Standard Taq Reaction Buffer with New England Biolab ThermoPol buffer. The third set of reagents used was from the QIAGEN Type-it microsatellite kit.). For this study, three primers sets were chosen: CUTC6, CUTC12, and CUTD107

The first method performed involved PCR amplification of each locus using the protocol outlined in Rasgon et al. (2006). Different reactions were set up to dry different combinations of reactions with all PCR reactions were 25 μL in volume. Each reaction contained 1.5 μL of DNA and 23.5 μL of master mix. The reagents used to create the master mix were 10X Standard Taq Reaction Buffer with $MgCl_2$, Nuclease-Free water, forward primer, reverse primer, dNTPs, and Taq DNA Polymerase. The primers were diluted to 100 μM. The volumes of reagents were changed throughout the reactions in order to determine what combination lead to the best clarity during gel electrophoresis. There was a range of reagents used (**Table 2**), with one set of thermocycler conditions (**Table 3**).

Table 1. Microsatellite loci used (Rasgon et al., 2006).

Locus	Primer	Core repeat	Size
CUTC6	F: 5'-GCGTTTGTCATCTGGTGG-3' R: 5'-GGGTTCGGAGCAGGAGTA-3'	$(ATG)_n$	216-228
CUTC12	F: 5'-GTGGAGAACCCGTATTCAAC-3' R: 5'-TACAATCACGACTCGCACATA-3'	$(ATG)_n$	184–211
CUTD107	F: 5'-ATGCCGACAGGGAGTTTC-3' R: 5'-CAAAGGTCTCACGACAGAGC-3'	$(CAG)_nCAA(CAG)_nCA(ACA)_n$	184–196

Table 2. Range of reagents used for the initial PCR experiments.

Reagent	Range tested
Primers (forward and reverse)	1.0-5.0 µL
dNTPs	1.0-5.0 µL
10x Standard Taq Reaction Buffer	2.0-5.0 µL
Taq DNA polymerase	0.5-2.0 µL

Table 3. Thermocycler conditions for CUTC6, CUTC12, and CUTD107 loci.

Differences for CUTD107 indicated in parenthesis.

Component	Time	Temperature
Initial heat activation	5 minutes	95°C
35 cycles of: Denaturation Annealing Extension	 60 seconds 60 seconds 30 seconds	 95°C 59°C (64°C—CUTD107) 72°C
Final extension	10 minutes	72°C

Thermocycler conditions were determined empirically with the base composition of the primer set (Rasgon et al., 2006).

For the next method, a different buffer, ThermoPol, was used in place of Standard Taq Reaction Buffer with $MgCl_2$. The rest of the conditions were identical. This setup was used for two runs: one testing site 253 DNA extractions (Ninepipe, located in Lake County) and one using site 702 DNA extractions.

Next, QIAGEN Type-it microsatellite PCR kit was used. As stated in the QIAGEN protocol, two reactions set-ups were used for this kit—one with the Q-Solution and one without the Q-solution (**Table 4**). The thermocycler conditions were determined using the QIAGEN Type-it microsatellite PCR kit protocol (**Table 5**).

Gel visualization was done on a 1% agarose gel with 1 µL of SybrSafe per 50 mL of agarose. Each lane contained 20 µL of the sample with a 100 base pair ladder in the first lane of each gel. The gels were run at 110 V for 20 minutes then visualized under Trans UV light.

Table 4. Reaction setup with Type-it microsatellite kit.

Component	**Volume/reaction**
Reaction Mix	
2x Type-it Multiplex PCR Master Mix	12.5 µL
Forward primer	2.5 µL
Reverse primer	2.5 µL
RNase-free water or Q-solution	5.0 µL
Template DNA	2.5 µL

Table 5. Thermocycler conditions for Type-it microsatellite kit.

Component	**Time**	**Temperature**
Initial heat activation	5 minutes	95°C
28 cycles of:		
Denaturation	30 seconds	95°C
Annealing	90 seconds	57°C
Extension	30 seconds	72°C
Final extension	30 minutes	60°C

DNA Quality

As further described in the results, amplification did not consistently occur, so the DNA was tested to see if it had potentially degraded. To test for DNA degradation, mitochondrial cytochrome c oxidase subunit I *(COI)* sequences were used. The adapted master mix solution outlined in Fiocchi (2016) was used (**Table 5**). The thermocycler protocol from Folmer et al. (1994) was used (**Table 6**).

Table 5. Master mix used for COI sequences.

Component	Volume/reaction
Reaction Mix	
Nuclease free water	31.0 µL
10x standard Taq reaction buffer	5.0 µL
25 mM $MgCl_2$	5.0 µL
10 mM dNTP mix	1.0 µL
Forward primer (LCO1490)	2.5 µL
Reverse primer (HCO2198)	2.5 µL
Taq DNA polymerase	1.0 µL
Template DNA	2.0 µL

Table 6. Thermocycler conditions used for COI sequences

Component	Time	Temperature
Initial heat activation		95°C
35 cycles of:		
Denaturation	1 minute	95°C
Annealing	1 minute	40°C
Extension	1.5 minutes	72°C
Final extension	7 minutes	72°C

Results

No amplification occurred with the CUTC6 and CUTD107 primers under any of the varying conditions. Different DNA samples were used to try to get some amplification, but these specific primers did not amplify any DNA (**Figure 1**).

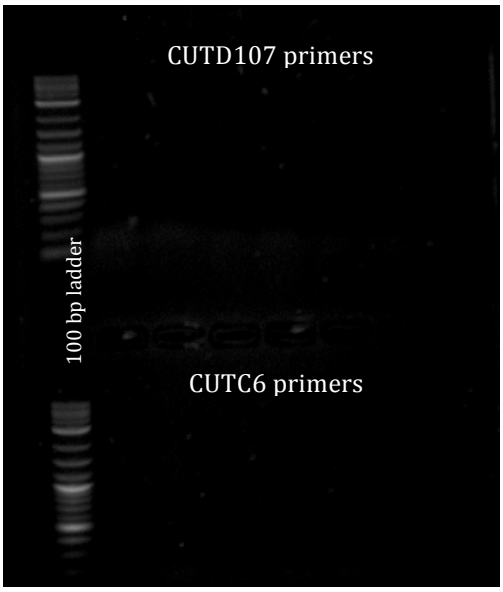

Figure 1. Top of gel is CUTD107 and bottom of gel is CUTC6 with a 100 base pair ladder in the first lane.

Amplification was achieved with the CUTC12 primer under the following condition: 3.0 µL of PCR buffer (with $MgCl_2$), 3.0 µL of dNTP mix, 2.0 µL of forward primer, 2.0 µL of reverse primer, 0.5 µL of Taq polymerase, 13.0 µL of nuclease-free water, and 1.5 µL of DNA. The DNA samples used were from site 253, which is Ninepipe #1 located in Lake County. Within this population, dilutions one through six were used (**Figure 2**).

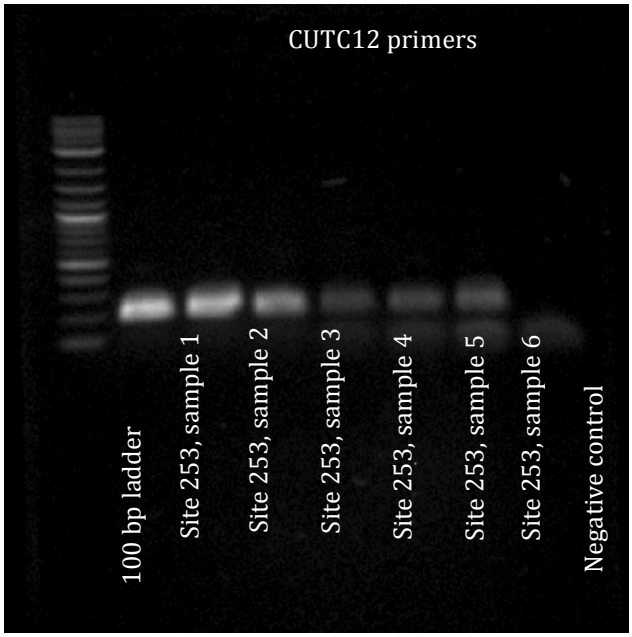

Figure 2. Gel of site 253 with CUTC12 primers. The first lane is a 100 base pair ladder and row 8 is a negative control (water). Lanes two through seven show amplification of the expected size, as listed in **Table 1**, from dilutions one through six respectively. The small amplified product in the negative control is likely primer dimer.

Subsequent experiments with the same conditions were run using different DNA samples. The positive control for these experiments was one of the samples from site 253 that previously amplified. No amplification was found in any of these runs. Thermocycler conditions were changed as were reagent volumes, but no amplification resulted. Likewise, the reactions set up using the ThermoPol buffer and the QIAGEN Type-it microsatellite kit also produced no amplification. Additionally, the *COI* sequence reactions produced no amplification.

Discussion

Microsatellites are particularly useful for genetic analysis over other methods, such as mtDNA, because microsatellites regions are highly variable as they are unstable and tend to have higher rates of mutation than mitochondrial DNA (Vieira et al., 2016). This, along with being nuclear markers, which are co-dominantly inherited, makes them ideal genetic markers for populations with a short generation time like mosquitoes. Microsatellites are easily transferred between individuals of related species. Often unequal crossing over leads to insertions or deletions, which results in differences in the number of repeats between individuals and populations (Vieira et al., 2016). Microsatellite analysis of *C. tarsalis* in Montana would be useful to create an accurate landscape genetic model by comparing the genetic structures of populations across the state to evaluate differences between populations where WNV is common versus where it is rare. The first step for creating this model is to develop a reliable microsatellite protocol. The primary goal of this study was to find the ideal reaction conditions for microsatellite analysis of *Culex tarsalis* in Montana populations. Using the primer sequences outlined in Rasgon et al. (2006), various aspects of PCR conditions were altered throughout this study.

No amplification occurred using the thermocycler conditions in Rasgon et al. (2006). In order to resolve this problem, the annealing temperature was lowered. With a lower annealing temperature, CUTC12 amplified while CUTD107 and CUTC6 still did not amplify. In the future, different annealing temperatures could be tested to try to get amplification with these two primer sets. However, this study continued with the

CUTC12 primer for further analysis. After amplification was achieved, the concentration of the reagents was altered until the desired clarity was reached.

As shown in **Figure 2**, amplification of the targeted region with CUTC12 was achieved during this study. However, even without changing the conditions of the PCR, the same results were not seen in other populations. There was no amplification in the positive control of these reactions. This indicates there is either a problem with the PCR setup or the DNA used has degraded. Because working primers are kept at low concentration, they do degrade, which could have been a possible cause of the PCR reaction no longer amplifying. However, when new working primers were made from the stock primers, the PCR still did not amplify. There is a possibility that the stock primers also degraded, which could be confirmed by obtaining new primers.

Another possible reason for the lack of amplification is the DNA polymerase. Each DNA polymerase has slightly different properties. *Taq* polymerase was used in this study, but further studies could use different DNA polymerases to see if a different DNA polymerase results in better amplification. A study done by Rocha et al. (2014) compared different DNA polymerases in microsatellite amplifications. This study used three different brands of polymerase and found Takara Advantage 2 Polymerase Mix is the most effective for microsatellite analysis. The original study of microsatellites in *Culex tarsalis* also used *Taq* polymerase as their DNA polymerase, however, they used ThermoPol buffer while this study used standard buffer (Rasgon et al., 2006). This buffer was ordered and used for subsequent reactions, but no amplification occurred when the Thermopol buffer was used. Additionally, the QIAGEN Type-it microsatellite kit did not result in any amplification. I was unable to test the Takara Advantage 2 Polymerase Mix.

Because no amplification occurred when changing reagents and reaction conditions, I hypothesized that perhaps the DNA had degraded during storage and/or freeze/thaw cycles. I tested for DNA amplification of *COI* sequences because they have successfully amplified in our *Culex tarsalis* DNA samples in the past (Fiocchi, 2016). A previous study by Shao, Khin, & Kopp (2012), showed that DNA begins to degrade after about 18 freeze/thaw cycles, depending on the original length. The ice crystals formed during freezing places tension on the structure of DNA. Because this DNA is from 2012, it has likely been through more than 18 freeze/thaw cycles. No amplification was seen using these primers, indicating the DNA used could have degraded. To test this hypothesis, further DNA extractions will need to be done.

This research was a continuation of previous work at Carroll College, where students identified a need for modifying the microsatellite protocol (McKeown 2011 and Casey 2013). Given the possible degradation of the stored DNA, future work at Carroll College should utilize fresh DNA from *C. tarsalis* populations to identify the reagents and reaction conditions for optimizing the microsatellite protocol. Published studies demonstrating that mountains and waterways can serve as barriers to gene flow in *C. tarsalis* (Venkatesen et al., 2007; Barker et al., 2009) highlight the efficacy of microsatellite analysis for comparing the genetic structure of mosquito populations across the state of Montana.

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