Using Microsatellite Loci to Analyze Genetic Variation of *Culex tarsalis* Within 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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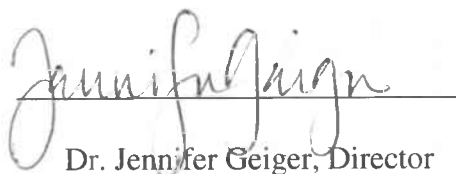

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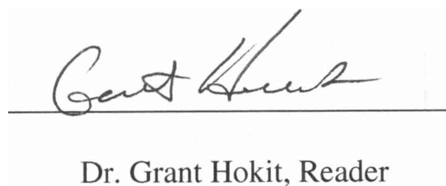

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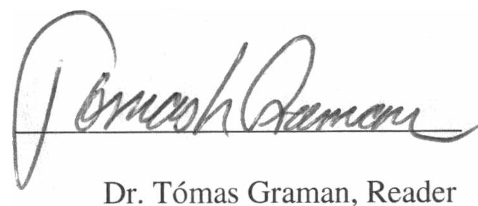

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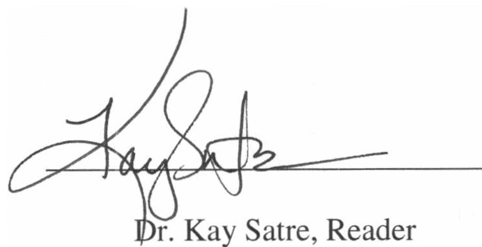

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Abstract

West Nile Virus (WNV) first arrived in the United States in 1999 and by 2002 was detected in Montana. The main bridge vector of WNV in Montana is the mosquito species *Culex tarsalis*. We are using geographic information system (GIS), molecular tools and landscape modeling techniques to develop a landscape-scale model of WNV infection risk for the state of Montana. The goals of this specific study were to identify microsatellite loci useful for characterizing the population genetic structure of *C. tarsalis* across Montana, and, utilizing this population genetic data, to infer migration (gene flow) patterns of mosquitoes across the state.

Utilizing two microsatellite loci, the distribution of genetic variation among and within five populations of *C. tarsalis* in Montana was preliminarily characterized. Through the genetic analysis of *C. tarsalis*, we hope to gain a better understanding of WNV ecology and factors affecting WNV distribution across Montana.

Introduction

Background

In 1999, West Nile Virus (WNV) was identified in the United States and in 2002 it was detected in Montana (CDC, 2002). WNV is an arbovirus (family Flavivirdae: *Flavivirus*) that is transmitted among avian hosts by mosquitoes, and in Montana, *Culex tarsalis* Coquillet (Diptera: Culicidae) is the primary bridge vector (Johnson and Hale, 2005). The amplification of WNV occurs during periods of adult mosquito blood-feeding by continuous transmission between mosquito vectors and bird reservoir hosts (CDC, 2009). Since WNV entered the United States, it has been responsible for large epidemics in humans, mammals, and birds (CDC, 2004). Infectious mosquitoes, such as *C. tarsalis*, store virus particles in their salivary glands and infect susceptible bird species when they bite them and take a blood-meal (CDC, 2009). At Medicine Lake in northeast Montana, the American White Pelicans, *Pelecanus erythrorhynchos*, are highly susceptible to WNV (Sovada et al., 2008).

The geographic range of *C. tarsalis* extends from the west coast of the United States to the Mississippi River and from northern Mexico to Canada (Darsie and Ward, 1981). Numerous studies have documented *C. tarsalis* as a major mosquito vector of WNV in many western United States (Turell et al., 2005), including California (Goddard et al., 2002; Reisen et al., 2004), North Dakota (Bell et al., 2005), and New Mexico (DiMenna et al., 2006). In 2003, the most prevalent transmission of WNV occurred in the western plains and Front Range of the Rocky Mountains, including Montana (Hayes et al., 2005). It was hypothesized that *C. tarsalis* was a major factor in recent epidemics

of WNV in the western United States, including Montana, because of its large population sizes and its efficiency for transmitting WNV (Hayes et al., 2005).

C. tarsalis has been found to be an efficient bridge vector for WNV because of its ability to amplify and maintain WNV (Goddard et al., 2002). It has been hypothesized that *C. tarsalis* could be the principal vector of WNV in rural agricultural ecosystems based on its vector competence and host-feeding patterns (Goddard et al., 2002). This finding has direct implications for Montana with its predominantly rural population. *C. tarsalis* is able to transmit WNV vertically, which means an infected female mosquito can pass the virus to her offspring (Goddard et al., 2003). Vertical transmission allows WNV to overwinter and to persist between infectious periods. Spatial variation in the ability of *C. tarsalis* to transmit WNV horizontally and vertically has been documented (Goddard et al., 2003). Spatial variation in vector competence that is correlated with genetic differences among populations exists in the flavivirus mosquito vector, *Aedes aegypti,* which is similar to *C. tarsalis* in its ability to transmit flaviviruses efficiently (Black et al., 2002).

Use of Genetic Markers

Numerous studies have found microsatellite markers to be useful for measuring genetic variation. Rasgon et al. (2006) and Venkatesan et al. (2007a) identified 12 and 45 novel *C. tarsalis* polymorphic microsatellite markers in *C. tarsalis*, respectively. Microsatellites are efficient for investigating population structure and constructing genetic maps in *C. tarsalis* (Rasgon et al., 2006). Microsatellite markers have been shown to be useful in examining the population structure dynamics of other mosquito

species, such as the WNV vector *Aedes taeniorhynchus,* and to determine the role of mosquitoes in the introduction and spread of novel pathogens (Bataille et al., 2009).

C. tarsalis is known to have significant spatial and temporal heterogeneity in numerous phenotypic traits such as feeding behavior, autogeny, and vertical and horizontal arboviral transmission (Venkatesan et al., 2007b). Microsatellites and other future genetic markers are predicted to be effective in investigating relationships between population structure and phenotypic traits (Rasgon et al., 2006) and should be valuable for studies investigating population structure and genetic mapping in *C. tarsalis* (Venkatesan et al., 2007a).

Spatial and Temporal Variation

C. tarsalis populations in California and Nevada have been shown to exhibit low temporal genetic variation, which may be due to seasonal fluctuations in *C. tarsalis* numbers or the immigration of genetically distinct individuals (Gimnig et al., 1999). The genetic structure of *C. tarsalis* populations is stable during the course of a season (Gimnig et al., 1999). However, the observed clustering patterns of *C. tarsalis* populations are dependent upon geographic features, such as mountain ranges (Gimnig et al., 1999). Mountain ranges or arid conditions that decrease the number of larval development sites seem to be important barriers to the dispersal of *C. tarsalis* (Gimnig et al., 1999).

Population genetic structure of *C. tarsalis* has also been studied in Colorado. Twenty populations of *C. tarsalis* along a riparian corridor following the South Platte River and Big Thompson River in northeastern Colorado were analyzed using five

microsatellite loci (CUTC6, CUTC12, CUTD107, CUTD113, and CUTD120, identified by Rasgon et al., 2006) and two single nucleotide polymorphism (SNP) loci (Barker et al., 2009). All collections from the ~190 km riparian section in northeastern Colorado were genetically uniform, but the populations were genetically separate from populations on the western slope of the Continental Divide (Barker et al., 2009). This pattern suggests that major waterways in the Great Plains serve as important dispersal passages for *C. tarsals*; however, the Continental Divide, which separates the eastern Colorado plains from the western high plateau, is a formidable barrier to this WNV vector (Barker et al., 2009). *C. tarsalis* had a prominent pattern of consistently high abundances between 1,200-1,450 m followed by a steady decrease in numbers above 1,450 m to reach very low levels above 1,550 m (Barker et al., 2009). *C. tarsalis* were most often infected with WNV in the plains portion of the riparian corridor (Barker et al., 2009).

Venkatesan et al. (2007b) used the same five microsatellite markers as the Barker et al. (2009) study, in addition to mitochondrial sequence data, to investigate the genetic structure of twelve *C. tarsalis* populations from California, Washington, Colorado, New Mexico, and Nebraska. Microsatellites uncovered moderate genetic structure and isolation by distance, whereas mitochondrial sequence data suggested panmixia across the region investigated (Venkatesan et al., 2007b). Microsatellite-based multilocus heterozygosity tests demonstrated a range expansion in most of the twelve *C. tarsalis* populations, which was estimated to have occurred across the western United States within the last 375,000-560,000 years (Venkatesan et al., 2007b).

Spread of Disease

In Montana, it is not understood if locations with high incidences of WNV, "hot spots," are due to recurring colonization incidents that would result in temporal variation or whether the genetic structure of the virus is such that sites close in geographic distance are more genetically related than distant sites, as postulated by Bertolotti et al. (2008). The locations of hot spots throughout the state are not clearly defined, but hot spots may be influenced by geographic, climatic and biological factors that establish the distribution of bridge vectors (*C. tarsalis* mosquitoes) and amplifying hosts, such as specific bird species (*P. erythrorhynchos)* (Theophilides et al., 2003). WNV ecology and evolution would be better understood through a genetic analysis of WNV and *C. tarsalis.* Furthermore, correlations between genetic structure and landscape geography could significantly improve risk assessment models. A definitive risk-assessment model for WNV in Montana would allow health officials and policy makers the ability to plan and allocate medical resources and surveillance activities in WNV hot spots. It would also allow scientists to understand distribution patterns of WNV better and possibly to predict potential patterns of expansion of the disease in the future.

I characterized the genetic structure of five *C. tarsalis* populations across Montana using two microsatellite loci (CUTD120, CUTD107: Rasgon et al., 2006). Migration and colonization patterns of the mosquitoes were preliminarily inferred to help explain how *C. tarsalis* may be affecting virus occurrence and spread.

The objectives of the study were to: 1) investigate and define the genetic structure of *C. tarsalis* populations across Montana, and 2) analyze the migration and colonization patterns of *C. tarsalis* in Montana. The null hypotheses were: 1) *C. tarsalis* has no

genetic variation among or within populations across the state, and 2) the patterns of migration and colonization of *C. tarsalis* are not limited by geographic features present in Montana or physiological limitations to migration; i.e., the Montana populations are panmictic.

Methods and Materials

The methods of Black et al. (2001) were used with the following modification to the DNA extraction protocol. DNA was extracted from 12-30 individual mosquitoes from five populations so that a sufficient statistical analysis of the genetic variation of the mosquitoes could be determined. To investigate and define the genetic structure of *C. tarsalis* populations in Montana, DNA was extracted from 93 mosquitoes from five sites in Montana (Fig. 1). Population locations, names, collection dates, numbers of individuals per population, and abbreviations are provided in Table 1.

According to the Black et al. (2001) DNA extraction procedure, I used a grinding buffer that consisted of (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 9.1, 0.05 M EDTA, 0.05% SDS). Grinding buffer was made up and stored frozen (-20°C) in 1 mL aliquots. Potassium acetate (8M) allowed for the removal of SDS from solution. Ethanol (100%) stored at -20°C precipitated DNA from solution. DNA was resuspended in TE buffer (0.01 M Tris-HCl pH 8.0, 1 mM EDTA) for long-term storage.

To prepare a 220 μL 10x primer mix with each primer at 2 μM, I started with 200 μL of nuclease-free water and added 5 μL of each of the two reverse primers, 2.5 μL of each of the two labeled primers, and 2.5 μL of each of the two unlabeled primers. I performed Multiplex PCR reactions at a total volume of 25 μL instead of 50 μL. I used an annealing temperature of 58°C and the number of amplification cycles was 30 (QIAGEN Multiplex PCR Kit Catalog No. 206143, 7/2004).

Amplification was confirmed via 1.5% agarose gel electrophoresis. Four microliters of PCR product were mixed with 4 μL of loading dye and the gels were run

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for 1 hour and 30 minutes at 73 volts. The gels were then stained for 30 minutes in an ethidium bromide solution and visualized under UV light.

When amplified product occurred, the ExoSAP-IT PCR Clean-up Protocol on page 6 of the ExoSAP-IT lab manual was followed with the following modifications: 2 μL of ExoSAP-IT were used per 21 μL of Multiplex PCR product. DNA concentrations of the PCR products were measured on a SmartSpec 3000 Spectrophotometer (Bio-Rad Laboratories, UK, Ltd). PCR products were diluted with nuclease-free water to the same DNA concentration. PCR products were sent to a GeneWiz, Inc. (South Plainfield, NJ) for genotyping analysis.

I used Applied Biosystems PeakScanner software (version 1.0) to score alleles for the two loci (*CUTD120* and *CUTD107*). I established a peak height minimum for alleles to be counted as viable alleles based on the relative size of the standard control peaks. I used FreeNA (Chapuis and Estoup, 2007) to estimate the frequency of null alleles, calculate genetic distances, and calculate F_{ST} values. I calculated allele frequencies, expected and observed heterozygosities, exact probabilities for Hardy-Weinberg and genotype disequilibrium, and F_{IS} values using GENEPOP version 4.0 (Raymond and Rousset, 1995). I used FSTAT version 2.9.3.2 to estimate allelic richness (Goudet 1995).

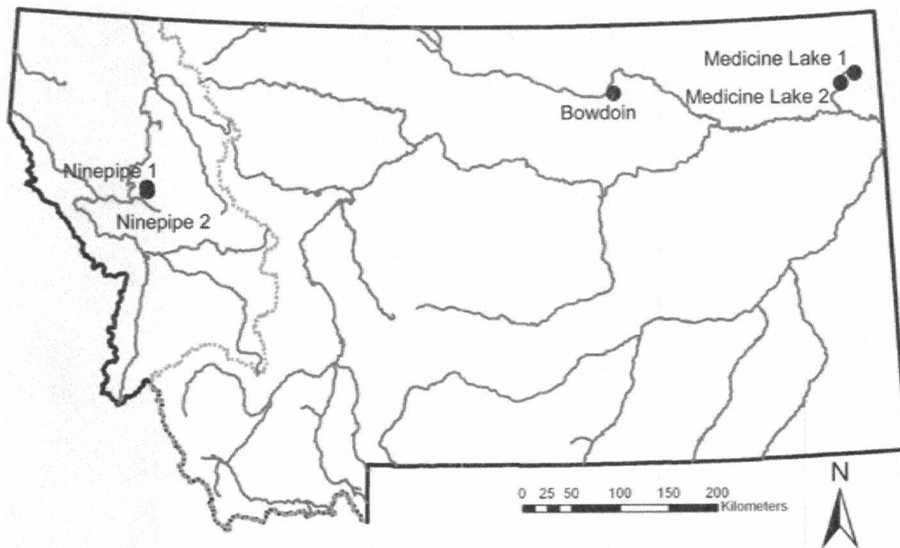


Figure 1. Map of Montana showing the five mosquito collection sites. The solid lines represent major rivers and the dashed line represents the continental divide.

Table 1. Collection sites of *C. tarsalis* analyzed in Montana.

Date	Location	County	# of mosquitoes	Abbrev
6/16/09	Ninepipe	Lake	20	NP1
7/15/09	Ninepipe	Lake	15	NP2
7/23/09	Medicine Lake	Sheridan	16	ML2
7/20/09	Medicine Lake	Sheridan	30	ML1
7/23/09	Bowdoin	Phillips	12	BD

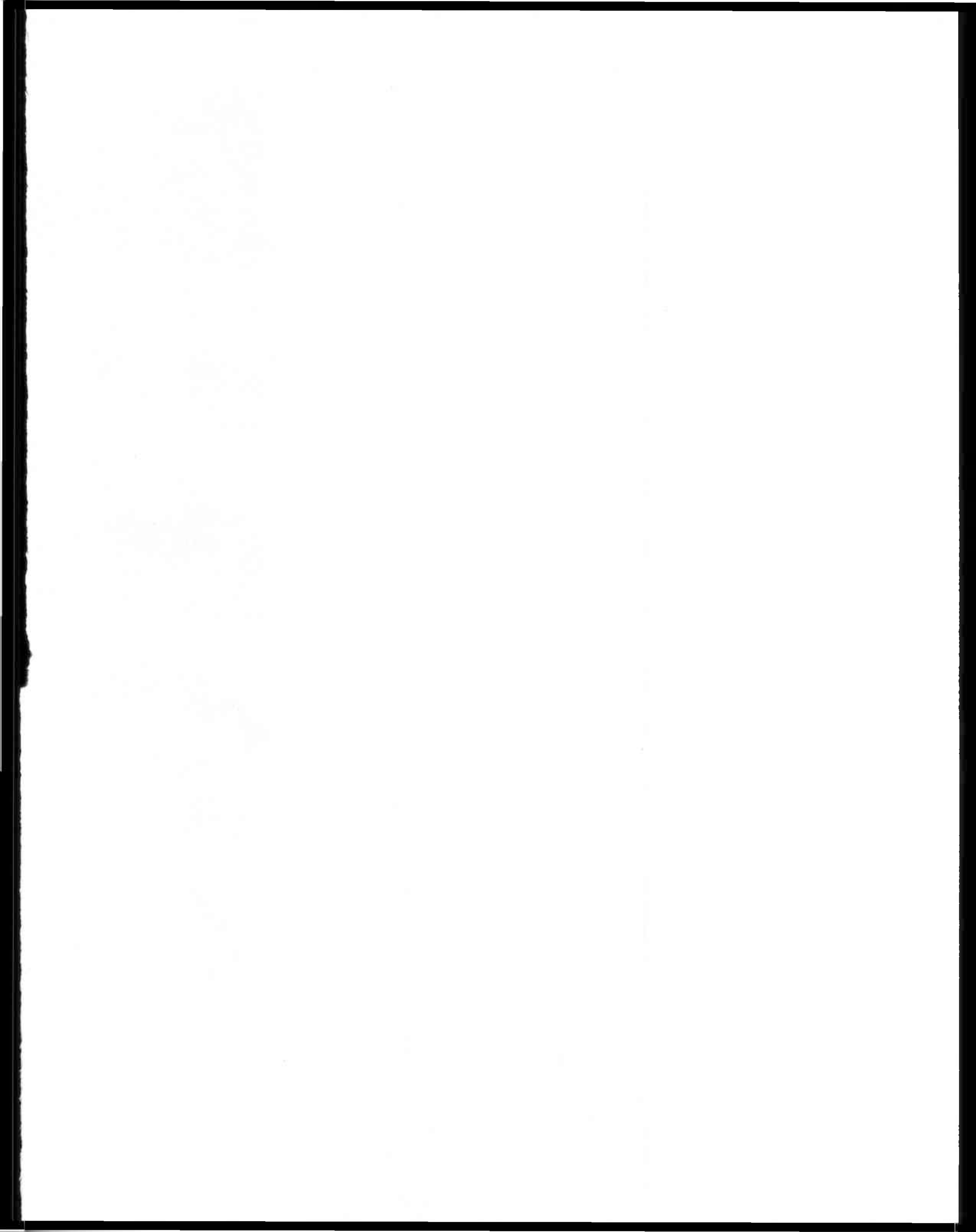
Results

Two microsatellite loci (*CUTD120 and CUTD107*) were amplified in Multiplex PCRs (Fig. 2) and successful genotyping of the loci occurred (Fig. 3). Some mosquitoes, such as ML1-1 and ML1-3, within the Medicine Lake #1 population were homozygous at the *CUTD107* locus (in blue), whereas other mosquitoes, such as ML1-3, were heterozygous at the *CUTD120* locus (in red) (Fig. 3). There were five alleles at the *CUTD120* locus and six alleles at the *CUTD107* locus, and the allelic frequencies in each population are given in Table 2. The null allele frequencies for the *CUTD120* and the *CUTD107* loci were 0.2315 and 0.0000, respectively. The allelic richness for the *CUTD120* and the *CUTD107* loci across all populations were 4.183 and 2.958, respectively.

The *CUTD107* locus is in Hardy-Weinberg equilibrium (HWE) for all five populations; however, the *CUTD120* locus is only in HWE for the ML1 population (Table 3). This is attributed to the presence of null alleles at the *CUTD120* locus. At the *CUTD107* locus, all five populations have F_{IS} values near zero, and at the *CUTD120* locus, all but the ML1 population had F_{IS} values greater than zero (Table 4). The ML1 and ML2 populations had the highest average expected heterozygosity (H_E) values, low F_{IS} values, and high levels of allelic richness (Table 4). The NP1 population had the lowest H_E value, the highest F_{IS} value, and the lowest allelic richness (Table 4). The number of mosquitoes analyzed in Table 1 and Table 4 are different because I was not able to acquire data from three of the mosquitoes in the ML1 population, four in the ML2 population, two in the NP2 population, and two in the BD population.

Populations in the same geographic region (ML1 & ML2 and NP1 & NP2) are most genetically similar, as measured by genetic distance (Table 5). Populations that are separated by large distances (ML1 and NP1) are the least genetically similar (Tables 5 and 6; Fig. 1). Populations that are separated by the least amount of geographic distance have the lowest F_{ST} values, whereas the populations separated by greater amounts of geographic distance have higher F_{ST} values (Table 5; Figure 1).

The 169 and 178 alleles were most prevalent at the *CUTD120* locus, whereas the 198 allele was most prevalent at the *CUTD107* locus, and these were present in all five populations (Table 2). The NP2 population was the only population with the 172 allele at the *CUTD120* locus, and NP1 and NP2 were the only two populations with the 186 allele at the *CUTD107* locus (Table 2). The ML1, ML2, and BD populations all had the 195 and 201 alleles at the *CUTD107* locus, whereas the ML1 and ML2 populations were the only two populations that had the 192 allele (Table 2).



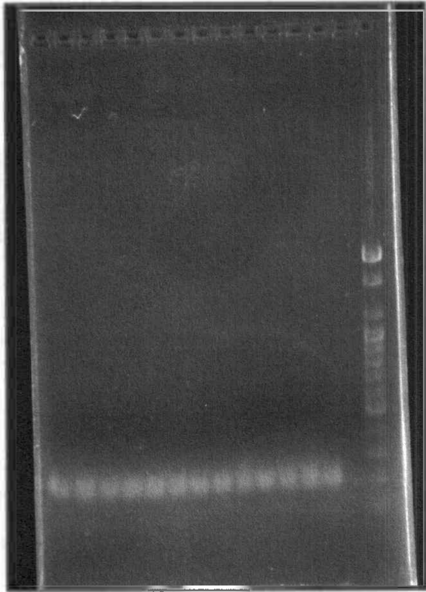


Figure 2. Multiplex PCR gel of DNA from Medicine Lake site #1. The desired two amplified products ranging from ~170-200 base pairs (bp) are evidenced in this gel. The second band from the bottom in the 100 kb ladder (last lane on the right), is a 200 bp standard fragment.

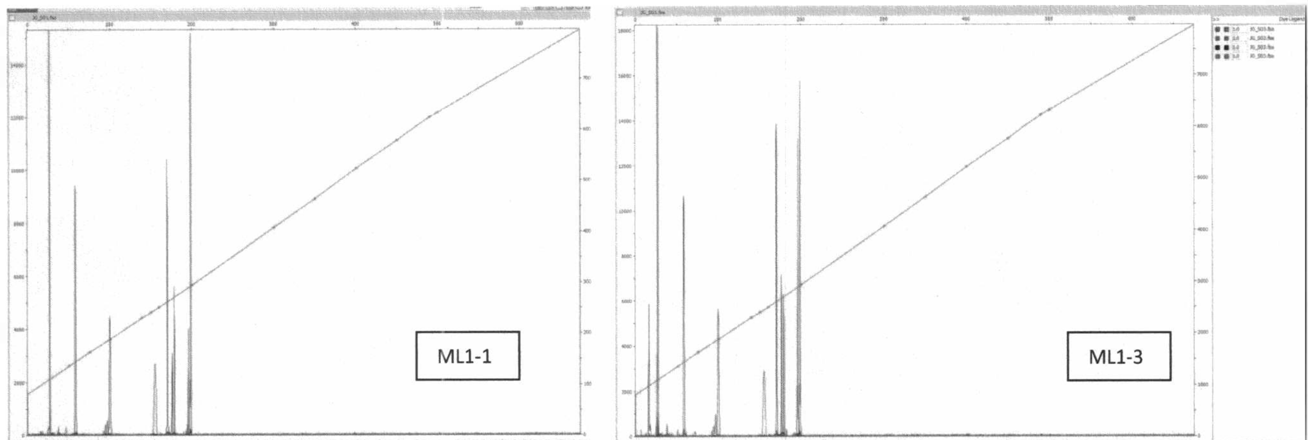


Figure 3. Two microsatellite loci genotypes from two mosquitoes (ML1-1 and ML1-3) from the Medicine Lake #1 population. Graphs show height of peaks plotted by base pairs. Orange peaks are standards; blue and red peaks represent different loci. Blue is the *CUTD120* locus and red is the *CUTD107* locus.

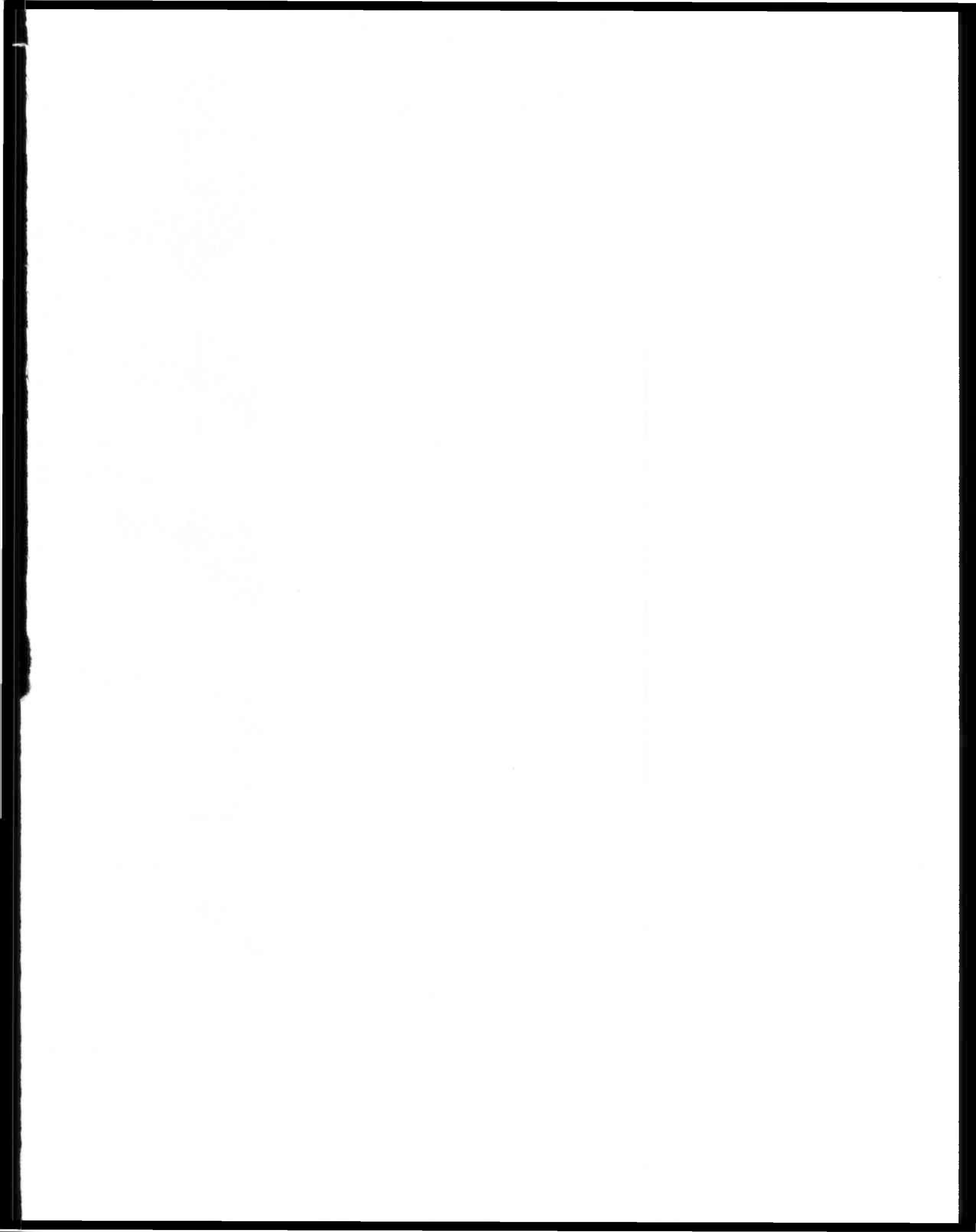


Table 2. Allelic frequencies for each locus in each population. See Table 1 for abbreviations.

Allelic Frequencies for Each Locus						
Locus: *CUTD120*						
	Alleles (# of base pairs)					
Pop	169	172	175	178	181	
ML1	0.426	0.000	0.185	0.333	0.056	
ML2	0.333	0.000	0.292	0.375	0.000	
NP2	0.154	0.154	0.231	0.423	0.038	
BD	0.550	0.000	0.050	0.300	0.100	
NP1	0.100	0.000	0.200	0.567	0.133	
All Pops	0.318	0.026	0.195	0.396	0.065	
Locus: *CUTD107*						
	Alleles (# of base pairs)					
Pop	186	189	192	195	198	201
ML1	0.000	0.033	0.017	0.150	0.783	0.017
ML2	0.000	0.000	0.031	0.094	0.813	0.063
NP2	0.033	0.067	0.000	0.000	0.900	0.000
BD	0.000	0.000	0.000	0.045	0.909	0.045
NP1	0.026	0.000	0.000	0.000	0.974	0.000
All Pops	0.011	0.022	0.011	0.071	0.863	0.022

Table 3. Genotype frequency tests for each locus for deviation from Hardy-Weinberg Equilibrium. See Table 1 for abbreviations.

Pop	Locus	p value
ML1	*CUTD120*	0.7549
	CUTD107	1.0000
ML2	*CUTD120*	0.0412
	CUTD107	1.0000
NP2	*CUTD120*	0.0000
	CUTD107	1.0000
BD	*CUTD120*	0.0088
	CUTD107	1.0000
NP1	*CUTD120*	0.0000
	CUTD107	1.0000

Table 4. Estimates of genetic diversity characteristics for each locus pooled across all five populations for 93 mosquitoes. H_E is the average expected heterozygosity, AR is the allelic richness, and N is the number of mosquitoes in the population. See Table 1 for abbreviations.

		Observed	Observed					
Pop	**Locus**	**Homozygotes**	**Heterozygotes**	**Total**	H_E	F_{IS} **Value**	**AR**	**N**
	CUTD120	8	19	27	0.704	-0.031		
ML1	*CUTD107*	17	13	30	0.433	-0.180	3.480	27
	All	25	32	57	0.561	-0.083		
	CUTD120	7	5	12	0.417	0.409		
ML2	*CUTD107*	10	6	16	0.375	-0.118	3.224	12
	All	17	11	28	0.393	0.239		
	CUTD120	9	4	13	0.308	0.598		
NP2	*CUTD107*	12	3	15	0.200	-0.050	3.665	13
	All	21	7	28	0.250	0.469		
	CUTD120	7	3	10	0.300	0.535		
BD	*CUTD107*	9	2	11	0.182	-0.026	3.409	10
	All	16	5	21	0.238	0.414		
	CUTD120	13	2	15	0.133	0.795		
NP1	*CUTD107*	18	1	19	0.053	0.000	2.744	15
	All	31	3	34	0.088	0.735		

Table 5. Genetic distances for *CUTD120* and *CUTD107* loci using the INA correction method for null alleles. See Table 1 for abbreviations.

Genetic Distances				
Dc using INA				
All loci				
Pop	**ML1**	**ML2**	**NP2**	**BD**
ML2	0.2343			
NP2	0.3751	0.3198		
BD	0.2558	0.2052	0.3178	
NP1	0.3757	0.2806	0.2017	0.2617

Table 6. Pairwise F_{ST} values for all five populations with corresponding geographic distances. Values above the diagonal are F_{ST} values, below the diagonal are geographic distance values. See Table 1 for abbreviations.

Geographic Distance Between Populations (km) and F_{ST} Values					
	ML1	**ML2**	**NP2**	**BD**	**NP1**
ML1	x	-0.001589	0.056176	-0.00129	0.081585
ML2	25	x	0.003864	0.02175	0.028059
NP2	740	730	x	0.065508	-0.013424
BD	250	240	490	x	0.096898
NP1	740	730	10	490	x

Discussion and Conclusion

The null hypothesis that *C. tarsalis* has no genetic variation within populations across Montana can be rejected because it has been shown that genetic variation exists within the populations (Tables 2 and 4). The *C. tarsalis* populations in eastern Montana (ML1, ML2, and BD) had a higher number of alleles on average than the *C. tarsalis* populations in western Montana (NP1 and NP2), suggesting a greater amount of genetic diversity in *C. tarsalis* in eastern Montana (Table 2). Allelic richness is similar in all five populations except for NP1, which is somewhat lower than the other populations (Table 4). The ML1 and ML2 populations have the highest H_E values and the lowest F_{IS} values, indicating a primarily outcrossing breeding system (Table 4). The number of alleles per locus and allelic richness is also high in the ML1 and ML2 populations (Tables 2 and 4), suggesting a high amount of genetic variation in *C. tarsalis* in the two Medicine Lake populations. The NP1, NP2, and BD populations have lower H_E values and higher F_{IS} values (Table 4), indicating inbreeding may occur in these populations and lower levels of genetic variation exist in these populations.

Genetic variation also exists among populations. Genetic distances and F_{ST} values (Tables 5 and 6) indicate that the two Medicine Lake populations are genetically very similar as are the two Ninepipe populations. The Bowdoin population is most genetically similar to the two Medicine Lake populations. ML1 and ML2 share a unique 192 allele at the *CUTD107* locus, and they share alleles 195 and 201 at the same locus with the BD population. In addition to being about 250 km away from the Medicine Lake populations (Tables 5 and 6; Fig. 1), the Missouri and Milk Rivers connect the Medicine Lake

populations to the Bowdoin population, so gene flow among *C. tarsalis* populations along this water corridor seems likely.

Among the population comparisons, the Ninepipe populations are the most genetically differentiated from the other populations sampled (Tables 5 and 6). The NP1 and NP2 populations share the 186 allele and were the only two populations lacking 195 and 201 alleles at the *CUTD107* locus, indicating some degree of genetic differentiation of those populations (Table 2). Since there is a great geographic distance (Table 6; Fig. 1) between the two Ninepipe populations and the other three populations, gene flow cannot occur as easily. Thus, as expected under a hypothesis of isolation by distance, these populations show genetic differentiation. The Continental Divide also separates the Ninepipe populations from Medicine Lake and Bowdoin populations, so if *C. tarsalis* mainly disperses along waterways, or movement of the mosquitoes is limited by altitude and mountain ranges as has been suggested (Gimnig et al., 1999; Barker et al., 2009), gene flow likely does not frequently occur between populations in eastern and western Montana.

C. tarsalis populations have been shown to exhibit low temporal genetic variation and the genetic structure of *C. tarsalis* populations appears to be stable during the course of a season (Gimnig et al., 1999). Consistent with this observation, the two Ninepipe populations that were collected one month apart had a very low F_{ST} value and the lowest genetic distance value of all pairwise population comparisons. Based on these data, *C. tarsalis* populations in Montana appear not to vary temporally in genetic structure, at least across a one month time period in this population.

The observation that *C. tarsalis* populations from eastern Montana were genetically similar, whereas the populations from western Montana were genetically distinct is not too surprising because of the existence of topographical factors in Montana that aid or prevent dispersal. The Milk and Missouri rivers in eastern Montana may allow for gene flow between *C. tarsalis* populations at Medicine Lake and Bowdoin. The Continental Divide, however, separates eastern and western Montana and is a significant barrier to dispersal of *C. tarsalis*. These results are consistent with those of the Barker et al. (2009) study done in Colorado, where they observed the Continental Divide to be a formidable barrier to *C. tarsalis*. They found that *C. tarsalis* collections from the eastern Colorado plains were genetically uniform while the collections on the western side of the Continental Divide were genetically distinct (Barker et al., 2009). Barker et al. (2009) also stated that their data for population genetic structure suggest that *C. tarsalis* disperses easily along rivers in the plains of eastern Colorado.

My results suggest genetic variation does exist within and among populations of *C. tarsalis* in Montana. Future studies may further quantify spatial and temporal diversity by utilizing additional microsatellite loci, a greater number of populations, and more frequent sampling during the summer season.

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