Genetic Comparisons of the Block Island Meadow Vole (*Microtus pennsylvanicus provectus*) to the Mainland Meadow Vole (*Microtus pennsylvanicus*) Based on Mitochondrial DNA

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Abstract – The Block Island meadow vole (*Microtus pennsylvanicus provectus*) is endemic to Block Island, Rhode Island, and listed as a Species of Greatest Conservation need by the Rhode Island Department of Environmental Management. The Block Island meadow vole has been designated as a subspecies based on morphological examination. We tested the subspecies classification by examining mitochondrial DNA (mtDNA) sequences from the cytochrome b (cyt*b*) gene of 25 Block Island individuals and 37 mainland individuals to formulate a more confident designation for the Block Island population and better direct management. We identified 1 mtDNA nucleotide that was unique to all Block Island meadow voles, which supports differentiation based on the Phylogenetic Species Concept. Sequencing results revealed 3 Block Island and 9 mainland mtDNA cyt*b* haplotypes. We suggest that the Block Island subspecies should continue to be treated as a separate management unit.

Introduction

Block Island (BI), which lies approximately 24 km off the coast of Rhode Island (RI), was created following a morainal drift deposited following the last glaciation about 20,000 years ago (Rosenzweig et al. 2000) (Fig. 1). The distance and time of separation have provided the opportunity for evolutionary processes to possibly lead to genetic differentiation of BI species from those that occur on the mainland (Plante et al. 1989). There has been some evidence of morphological differentiation in the Block Island meadow vole (*Microtus pennsylvanicus provectus*; BIMV) compared to the mainland meadow vole (*Microtus pennsylvanicus pennsylvanicus*; MMV), as reported in previous studies (Bangs 1908, Chamberlain 1954, Lowry 2002, Reich 1981, Tamarin 1985,). While voles are known to swim or otherwise cross waterways using floating debris, this has only been recorded for relatively short distances (Lowry 2002). Because of the great distance between the mainland and BI and the length of time that they have been separated, significant genetic differentiation between the 2 populations may have occurred.

These morphological studies used to examine the BI and mainland populations, resulted in the naming of the BI subspecies as *M. p. provectus* (Bangs 1908, Chamberlain 1954, Lowry 2003, Tamarin 1985). The naming was debated due to the lack of confidence in the technique utilized (Chamberlain 1954). Today, the BIMV population is treated generally as a subspecies and is listed among the Species of Greatest Conservation Need in Rhode Island by the Rhode Island Department of Environmental Management due to its limited geographic distribution (Carcieri et al. 2005).

Genomic DNA provides the opportunity to examine adaptive genetic variation beyond the limitations of thee biological species concept which delineates species as a result of reproductive isolation (Funk et al. 2012, Vogler and Desalle 1994). This concept relies upon the

designation of morphological traits among individuals in order to create groups, which does not always hold true as these can vary greatly. In order to overcome this issue the phylogenetic species concept utilizes genomic DNA to determine heritable characters in order to define a taxa or clades. As such, the smallest group of organisms containing a unique character or combination of characters is grouped together (Vogler and Desalle 1994). From which, these groups can be designated as a conservation unit for the use of management.

In a similar study conducted by Lowry in 2002, morphological evidence, mtDNA and, microsatellites were utilized to determine the taxa of meadow voles found throughout islands of Penobscot Bay, Maine. Both microsatellites and mtDNA were used to reject exchangeability, or accept the designation as an evolutionary significant unit, and mtDNA was used to determine the historical lineage. All supported the differentiation of the island species *M. p. shattucki* from *M. pennsylvanicus*. This study looks to accomplish similar designations of the BIMV through the use of genetic evidence.

We examined sequences from the mitochondrial DNA cytochrome b gene of MMV (n=37) and BIMV (n=25) individuals. We tested the hypothesis that there is a significant genetic difference to differentiate *M. p. provectus* as a subspecies or separate species from *M. pennsylvanicus* and reject the null if there is no significant genetic difference. Along with this, we looked to create an ecological history to determine a time of separation and source of genetic material. We hypothesize that the original parent haplotypes may have been isolated at the time of the last glaciation, about 20,000 years ago (Heckel et al. 2005). In recent decades, increasing pressures from human activities have impacted a number of species on BI. The goal of our study is to determine the taxonomic status of the BIMV by examining the genetic and evolutionary

history of this species. Findings of our work will inform management decisions regarding designation of the BIMV as a specific conservation unit.

Field-site Description

BI is situated approximately 19.3 km south of Point Judith, Rhode Island, and 22.5 km east of Montauk Point, Long Island, New York. The island consists of rolling hills adjacent to the shoreline, which is laden with beach grasses along with upland shrub habitat. There are 2 large ponds called Sachem Pond, which is a northern brackish pond, and Great Salt Pond, a saline pond located on the western side of the island. Along with these 2 water bodies are hundreds of small ponds and interconnected wetlands (Rosenzweig et al. 2000).

Sampling occurred in fields of switch grass (*Panicum virgatum)* where traps were placed in or near visible *Microtus* trails. These fields were often associated with a body of water or a wetland, which is the preferred meadow vole habitat.

Methods

Sampling

Voles were trapped at 8 sites, all of which were encompassed by stonewalls, commonly used to define property boundaries. We employed Sherman traps, which were baited with peanut butter and provisioned with cotton for bedding. Traps were checked twice daily and all trapping

followed the guidelines of the American Society of Mammalogists (Sikes et al. 2011) and were approved by the University of Rhode Island (URI) Institutional Animal Care and Use Committee (AN1314-002). Blood was collected from all captured individuals using FTA cards after nicking the saphenous vein with a diabetic lancet. Standard measurements were taken for all individuals, after which they were ear tagged and released into their immediate environment. Deceased individuals were kept on ice until stored in a -80ºC ultra-low freezer. Ten individuals from BI were examined.

Mainland meadow voles were captured and euthanized by members of the U.S. Fish and Wildlife Service (USFWS), as part of a disease study at the Ninigret National Wildlife Refuge in Charlestown, Rhode Island. Necropsies of each individual were conducted at the Roger Williams Park Zoo in Providence, RI. Tissues samples were taken from 18 individuals (ear, skin, and tail) and stored in 100% ethanol for later genetic analysis at the University of Rhode Island's Wildlife Genetics and Ecology Laboratory.

In addition 17 BIMV and 19 MMV samples from a previous study conducted at URI in 1998 were also examined (Husband personal communication). These samples were liver tissue that was extracted using a Qiagen® kit. In total, 62 individuals were examined, of which 25 originated from Block Island and 37 from mainland RI.

DNA extraction and mitochondrial DNA sequencing

Genomic DNA was extracted from 0.2 g of tissue using a NucleoMag® tissue extraction kit (Macherey-Nagel Inc., PA) following the manufacturer's instructions. Two punches from each FTA card were extracted using DNeasy® blood and tissue kit (Qiagen, CA) following the

manufacturer's instructions. A NanoDrop 1000 spectrophotometer was used to quantify the amount and quality of DNA in the samples acquired from 1998 (Thermo Scientific, MA).

We amplified 995 base pairs for the cyt*b* of both MMV and BIMV through polymerase chain reactions (PCR). Amplifications of mtDNA, PCR contained 7 µl of water, 2.5 µl of Bovine serum albumin (BSA), 1 µl of the forward primer L14727-sp (Jaarola and Searle 2002) (10 pmol), 1 µl of the reverse primer H15915-sp (Jaarola and Searle 2002) (10 pmol), 12.5 µl of Top Taq polymerase (Qiagen, CA) and 1 µl of the DNA extraction for the tissue extractions. The blood samples were cleaned up using NucleoSpin® gDNA Clean-up XS (Macherey-Nagel Inc., PA) following the manufacturer's instructions. Blood sample PCR reactions contained 4 µl of water, 2.5 µl of BSA, 1 µl of the forward primer L14727-sp (Jaarola and Searle 2002) (10 pmol), 1 µl of the reverse primer H15915-sp (Jaarola and Searle 2002) (10 pmol), 12.5 µl of Top Taq polymerase, and 4 µl of DNA extraction.

Tissue PCR products were performed on the Eppendorf Mastercycler EP Grandients S (Eppendorf, Germany). The samples were denatured for 1 minute at 94C followed by 30 cycles of denaturation at 94C for 30 seconds, annealing at 52C for 30 seconds, extension at 72C for 30 seconds, and a final extension at 72C for 10 minutes. Blood samples were denatured for 1 minute at 94C followed by 30 cycles of 1 minute denaturation at 94C, 30 seconds of annealing at 48C, 30 seconds of extension at 72C, and a final extension for 10 minutes at 72C. Following amplification 5 µl of all samples were run on an agrose gel with 3 µl of SYBR® Safe DNA Gel stain (life technologies, NY) to and 4 µl of ladder. The electrophoresis was run for 50 minutes at 110 volts. Carestream (Carestream Health inc., Canada) was used to visualize the gel. Lastly, all samples were purified using Agencourt AMPure XP using 12 µl of bead, 20 µl of PCR product, and 8 µl of water.

Sanger sequencing (Sanger et al. 1977) was performed by the Genomics and Sequencing Center at the University of Rhode Island. To visualize sequences and trim sequence ends we used Geneious software (Biomatters Ltd., Auckland, New Zealand). The sequences were aligned using the Clustal W algorithm (Thompson et al. 1994) and gaps and uncertainties were corrected visually. We also included 24 mtDNA cyt*b* sequences from Genbank (table 1).

Genetic analyses

MEGA was used to determine distance and diversity measurements using 1000 bootstrap replications. We used the Kimura 2-parameter model in MEGA (Kumar et al. 2004). MrModeltest2 (version 3.06) (Nylander 2004) was used to choose the best fit substitution model for *cytb*. Phylogenetic analysis of cyt*b* sequences were done using PAUP* (version 4.08b) (Swofford 2002). The program MrBayes (Huelsenbeck and Ronquist 2001) was used to conduct Bayesian inference analyses using the best fit model GTR+I+G. The Markov chain Monte Carlo algorithm (MCMC) was run, sampled every 1000 generations and finished once the average standard deviation of split frequencies were <0.01. All chains beforehand were removed as a burn-in and the rest used to construct a consensus tree. Bayesian analyses were used to create a final phylogenetic tree which was generated following 3 independent runs. Confidence levels were averaged from these 3 runs and the standard deviation calculated for each group. TCS (Clement et al. 2000) was used to determine nucleotide base changes among haplotypes.

Results

9 MMV haplotypes and 3 BIMV haplotypes were defined. Differentiation between all haplotypes was low when intraspecific genetic distance and diversity was compared in where all BIMV haplotypes were grouped together and compared to all MMV haplotypes grouped together. MEGA determined the mean group distance within BI and ML haplotypes to be 0.005 ± 0.001 for ML and 0.002 ± 0.001 for BI. The group mean distance between resulted in 0.002 distance for ML and 0.005 for BI. The net between group mean distance resulted in both haplotypes having a distance of 0.001. The mean diversity within subpopulations was 0.003 ± 0.001. The mean interpopulational diversity was 0.001 ±0.001. All measures of distance and diversity resulted in similar values providing confidence in the intraspecific genetic distance and diversity.

The same was done in order to compare interspecific genetic distance and diversity among and between RI haplotypes and Alaska (AK) *M. p. pennsylvanicus* haplotypes (Hope et al. 2013, Kholi et al 2014) haplotypes. The RI group included both BIMV and MMV samples. The mean group distance within RI and Alaska was 0.005 ± 0.001for both RI and AK. The group mean distance between both haplotypes was 0.008 for RI and 0.042 for AK. The net between group mean distance was 0.008 for RI and 0.036 for AK. The mean diversity within subpopulations was 0.005 ±0.001. The mean interpopulational diversity was 0.017 ±0.004. Interspecific genetic distance and diversity was expectedly greater than intraspecific distance and diversity but still low.

We then compared RI to *M. townsendii* and *M. montanus*, the next 2 most closely related clades included in this study (Table 2). Distance just about doubled between RI and *M. montanus* haplotypes and between *M. montanus* and *M. townsendii* haplotypes which agrees with the phylogenetic tree (Fig. 2). The greatest amount of distance and diversity was apparent when

comparing interspecific genetic distance and diversity of RI haplotypes and *M. townsendii* and *M. montanus*. This though was not large enough to deem intraspecific genetic comparison levels too low to support separation among BIMV and MMV. As a result, while intraspecific comparison levels are low, they support differentiation.

The Bayesian Inference phylogenetic trees created by MrBayes grouped both MMV and BIMV haplotypes into a well-supported clade. RI haplotypes are further separated from AK *M. p. pennsylvanicus* and all other Microtus genera. Within the clade all BI haplotypes group together with a good bootstrap value of 74.3±.57. This suggests that there is separation between ML haplotypes and BI haplotypes but not enough for BI haplotypes to form a separate clade. There is little structuring amongst ML haplotypes, similar to results Lowry found (2002).

TCS defined the amount of changes when comparing intraspecific genetic haplotypes (Fig. 3). BI haplotypes were separated out from ML haplotypes by 1 variable site with a T nucleotide base changed to a C nucleotide base at position 320 (Fig. 4). This was consistent among all samples in where all MMV samples had a T nucleotide base where BIMV samples had a C nucleotide base. There were no differences when present day 2014 samples were compared to samples from 1998, so instead groups were separated by geographic location only. The majority of BIMV samples fell within BI haplotype 1 which was 1 nucleotide base different from the most closely related ML haplotype. As shown in Figure 1., individuals from BI haplotype1 were found on the majority of sites trapped around the island whereas BI haplotype 2 was only found at site 8. A large group of MMV samples fell within ML haplotype 4 which is 2 nucleotide bases different from that of BI haplotype 1. All haplotypes include few changes with 6, the most nucleotide base changes, occurring between ML haplotype 1 and ML haplotype 9.

The standard mtDNA sequence divergence rate per million years (pmy) is 2% for mammals. It has been stated that this divergence rate may be much higher in mammals (Kocher et al. 1989). Brunhoff et al. suggests that this may be about 3 to 5 times higher in *Microtus* cyt*b* than the standard mammal rate, instead applying a moderate 6-10% molecular clock (2003). It is then expected that 20,000 years will result in 0.0012-0.002 divergence. We used net between group mean distance calculated by MEGA. Both BI haplotypes and ML haplotypes had a 0.001 net between group mean (Brunhoff et al. 2003).

Discussion

Both Lowry and this study result in the distinction of genetic separation using mtDNA. Of which it was determined that BIMV and *M. p. shattucki* fell within a monophyletic clade (2002). microsatellites further corroborated Lowry's study which focused on islands in Penobscot Bay, Maine. Individuals found on the island of North Haven were found to be more genetically distant as a result of greater geographical distance and less human impact. It is then likely that the BIMV population may be susceptible to the same pressures. As a result, both may experience genetic distance resultant from glaciation.

The given evidence supports the fact that the BIMV population is genetically different from the MMV population. The 1 evident variable site was consistent among all BIMV samples. All statistical analysis supported this conclusion. The phylogenetic tree created using MrBayes separated the BIMV population from that of the MMV population. But the BIMV still shows relation to this MMV group in that it does not form a new clade, but rather creates an extension of MMV.

Historical context

The typical mtDNA standard rate of sequence divergence for mammals is 2% pmy (Brunhoff et al. 2003). This has been widely debated when applied to *Microtus* cyt*b* with some data suggesting that the evolutionary rate may be greater (Conroy and Cook 2000, Kocher et al. 1989). Using a moderate clock of about 6 to 10% agreed with the idea that the separation between the 2 populations may have been a result of the last glaciation period, the Laurentide ice sheet, about 20,000 years ago (Brunhoff et al. 2003). The melting of the glacier and resultant sea level rise led to the ocean barrier between the 2 land masses (Youngman 1967). Following this separation, there would have been little to no opportunity for connection due to the great distance between BI and the ML. This distance would have been too great for *Microtus* to swim, as they have a known swimming range of about 1 km (Lowry 2002). The most recent possible source of mixture would have been human dispersion. Despite this possible source of colonization or mixture, our study shows that there is enough genetic differentiation to conclude that glacial retreat may be the source of separation and that there has been no recent connection for this species.

Conservation implications

Due to a resultant uncertainty that has stemmed from the conclusions of previous research that designated the BIMV populations as *M. p. provectus*, conservation and management efforts have been relatively small in scale. There have been 3 factors identified as pressures limiting the BIMV population: natural succession of vole habitat, predation from housecats, and residential development (Carcieri et al. 2005). Planned action to deal with natural

succession involves management for early successional habitat (Francl et al. 2008) through the cooperation of private landowners to modify land practices, as well as, continuing to protect current habitat, which is essential to maintain genetic variation (Gauffre et al. 2008,Marchi et al. 2013). Management to address the issue of domestic cat predation would focus on educating the public and increasing programs to neuter domestic and feral cats. The resultant actions lack specificity and can be better directed towards the BIMV population if there is greater confidence in the delineation of a subspecies.

The 3 necessary tools for defining separation are morphological differences, geographic separation, and DNA evidence (DeSalle et al. 2005). In this study geographic separation is resultant of the ocean barrier between landmasses which halts immigration. Morphological differences among BIMV and MMV have been listed in previous studies, which lead to the eventual distinction of BIMV as a subspecies. It is the DNA evidence that this study provides that suggests the formation of a new conservation unit. It is suggested that BIMV be treated as a Management Unit (MU). An MU is defined as the management of a local population as a distinct unit because of demographic independence in where growth rate is resulted from local birth and death rates rather than immigration (Funk et al. 2012). This is used in cases of intraspecific designation. BIMV warrants its own management plan separate from that of MMV. To further corroborate this, nucDNA should be incorporated in future research in conjunction with our evidence based on mtDNA. When developing a conservation management plan for the BIMV population, it may be important to consider specific needs of this MU.

Acknowledgements

We thank Scott Comings, Director of Land & Freshwater Conservation for the Block Island Office of the Nature Conservancy for helping with field research and trapping on Block Island. We are grateful to the USFWS and Cynthia Maynard for collecting and providing mainland samples. We also thank the Roger Williams Park Zoo and Dr. Michael McBride for securing and preparing mainland samples. Mary Sullivan provided assistance with sequencing and statistical analysis. We are thankful those who permitted access to the property for sample collection. Funding and technical resources were provided by the University of Rhode Island Wildlife Genetics and Ecology Laboratory. This research is based in part upon work conducted using the Rhode Island Genomics and Sequencing Center which is supported in part by the National Science Foundation under EPSCoR Grants Nos. 0554548 & EPS-1004057.

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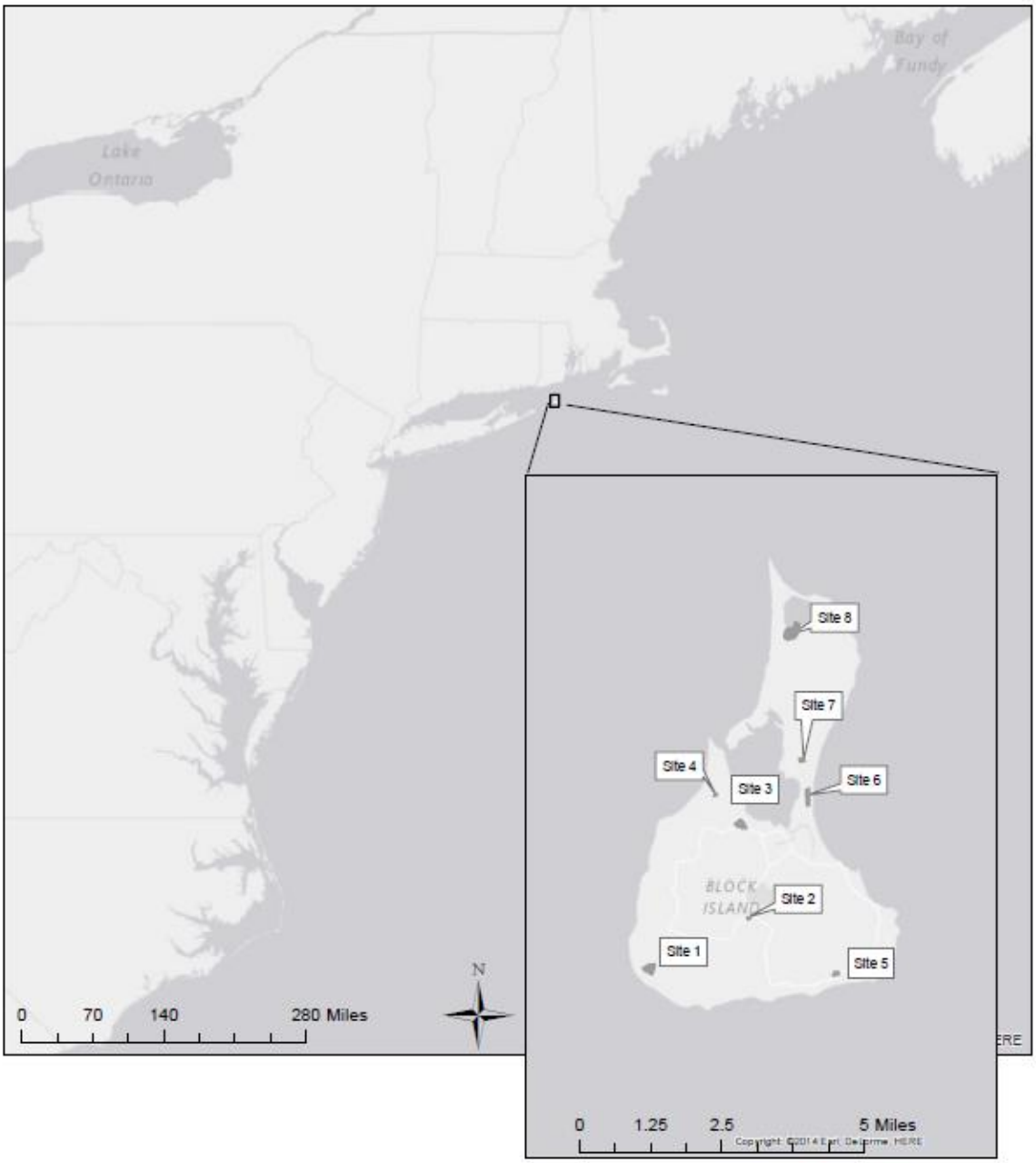

Figure 1. Map of BI trapping sites. BI haplotype 1 was found at site 1,2,4,6,7, and 8. BI haplotype 2 was only found at site 8. The location of BI haplotype 3 is unknown.

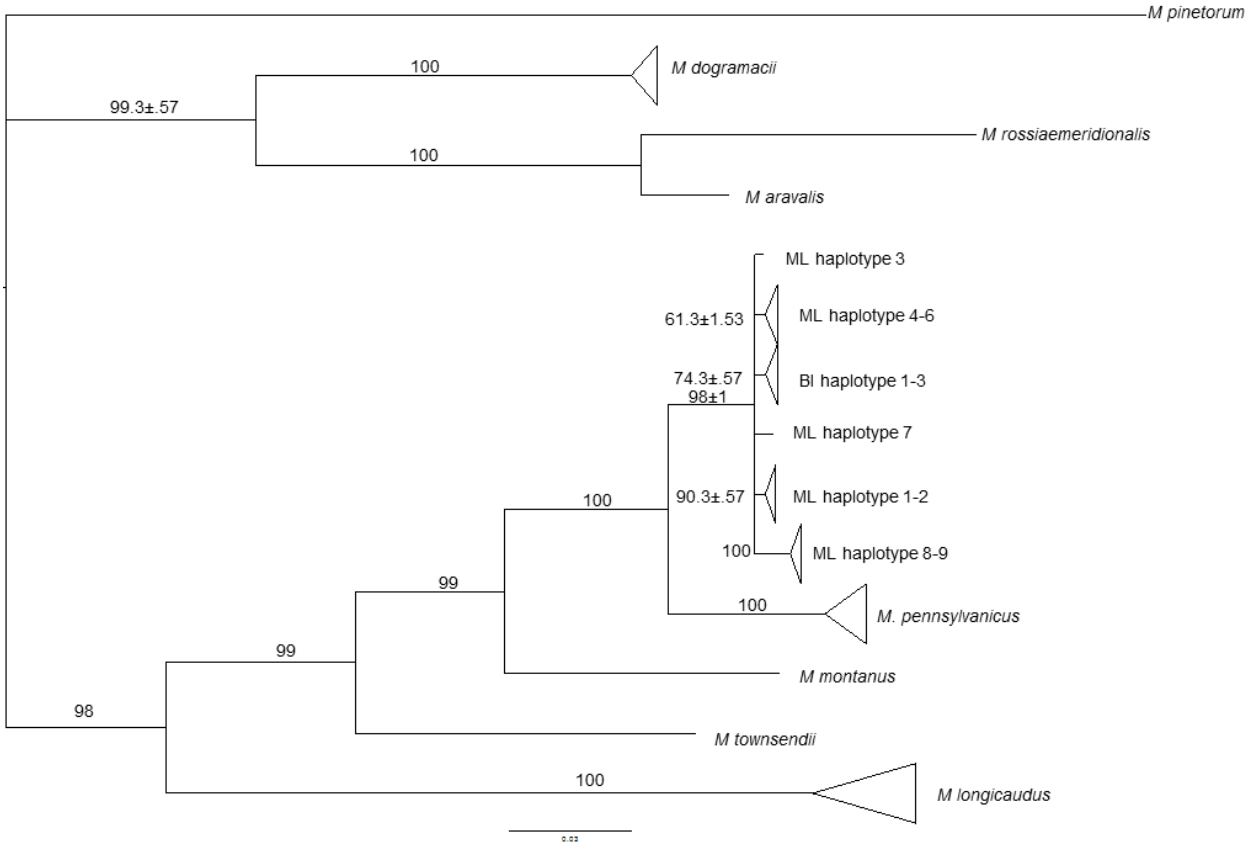

Figure 2. A Bayesian inference of cyt*b* sequence haplotypes of *Microtus spp*.

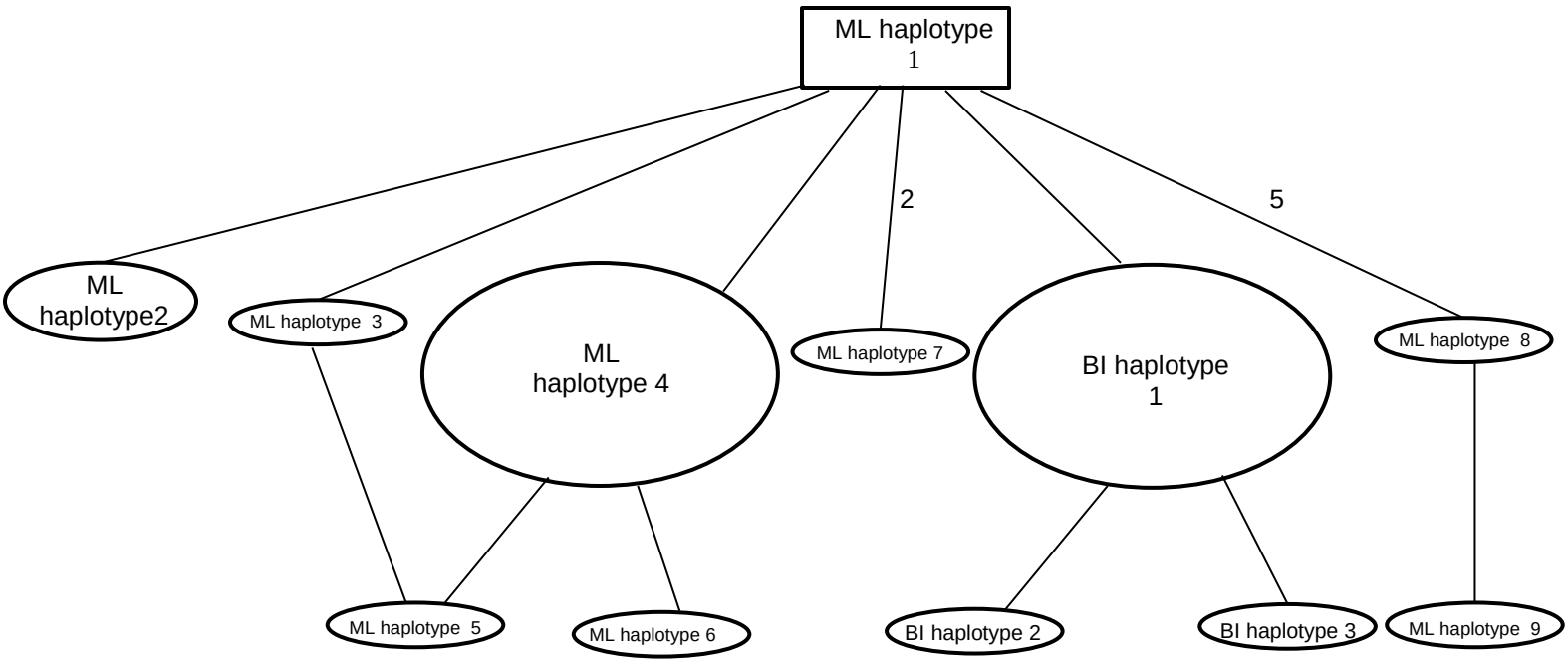

Figure 3. Network analysis of nucleotide base changes between haplotypes created using TCS (Clement et al. 2000). The size of each figure designates the number of samples that fall into a haplotype and a rectangle indicates the ancestor lineage. The number of changes in a sequence between 2 haplotypes is placed next to a connecting line and no number indicates 1 change. BI haplotype 1 has 20 BIMV sample, BI haplotype 2 has 2 BIMV samples, BI haplotype 3 has 2 BIMV samples, ML haplotype 1 has 3 MMV samples, ML haplotype 2 has 6 MMV samples, ML haplotype 3 has 2 MMV samples, ML haplotype 4 has 19 MMV samples, ML haplotype 5 has 1MMV samples, ML haplotype 6 has 1 MMV sample, ML haplotype 7 has 1 MMV sample, ML haplotype 8 has 2 MMV samples, Haplotype 9 has 3 MMV samples.

					1	1	1	1	2	3	3	4	4	5	7
	2	4	4	9	2	4	5	6	3	2	5	1	3	7	1
haplotype	9	1	4	8	9	0	8	0	0	0	9	3	8	0	7
ML haplotype 4	A	C	A	C	A	T	A	T	T	T	G	C	A	G	T
ML haplotype 6	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
ML haplotype 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
ML haplotype 7	.	T	G	.	.	.	.	.	.	.	.	.	G	.	.
BI haplotype 2	.	T	.	.	.	.	.	.	.	C	A	.	.	.	.
BI haplotype 1	.	T	.	.	.	.	.	.	.	C	.	.	.	.	.
ML haplotype 3	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C
ML haplotype 9	G	T	.	T	.	.	G	.	C	.	.	T	.	A	.
ML haplotype 1	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
ML haplotype 2	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.
BI haplotype 3	.	T	.	.	.	.	.	A	.	C	.	.	.	.	.
ML haplotype 8	G	T	.	T	.	.	G	.	C	.	.	.	.	A	.

Figure 4. Nucleotide base variation among BI haplotypes and ML haplotypes. Highlighting is used to display the 1 variable site apparent in all BIMV samples and BI haplotypes.

Table 1. GenBank accession numbers, location data, and articles referenced from for each sample used in the creation of a phylogenetic tree. Alaska samples are further broken down into subspecies based on the geospatial coordinates and the *M. p. pennsylvanicus* subspecies range map (Reich 1981).

Specimen/GenBank Accession no.	Location	Article reference	
M. longicaudus			
AF119267	Yakutat, AK	Conroy and Cook 1999	
AF187160	Jacob Lake, AZ	Conroy and Cook 2000	
M. montanus			
AF119280	Wood Hollow, UT	Conroy and Cook 1999	
M. townsendii			
AF163906	Prairie Mountain, OR	Conroy and Cook 2000	
M. dogramacii			
AY513793	Prairie Mountain, OR	Jaarola et al. 2004	
AY513794		Jaarola et al. 2004	
M. rossiaemeridionalis			
AY513819	Kauhava, Finland	Jaarola et al. 2004	
M. aravalis			
GU197787	Skara Brae, Mainland	Martinkova et al. 2013	
M. pinetorum			
AF163904	Pulaski County, AR	Conroy and Cook 2000	
			M. p. alcorni
KC473494	Chilkat River Valley, AK	Hope et al. 2013	*M. p. drummondii*
KC473477	S of Pelly Crossing, Yukon Territory	Hope et al. 2013	*M. p. alcorni* *
KC473491	West of Paxson, AK	Hope et al. 2013	*M. p. alcorni*
KC473492	Tiekel River, AK	Hope et al. 2013	*M. p. tananaensis*
KC473473	North of Minto, AK	Hope et al. 2013	*M. p. drummondii*
KC473476	Dawson City, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473478	S of Pelly Crossing, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473485	Minto Lake, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473484	South of Keno, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473488	North Fork of Klondike River, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473480	Fox Creek, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473489	North of Rock River, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473495	Stikine River at Hudson Flats, BC	Hope et al. 2013	*M. p. drummondii*
KC473490	North of Rock River, Yukon Territory	Hope et al. 2013	*M. p. drummondii* *
KC473482	Lake Laberge Campground, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473487	West of Stewart Crossing, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KC473481	Lake Laberge Campground, Yukon Territory	Hope et al. 2013	*M. p. drummondii*
KJ556623	Campell Hwy, Yukon Territory	Kohli et al. 2014	*M. p. alcorni*
KC473470	Idavain Lake, AK	Hope et al. 2013	*M. p. alcorni*
KC473471	Idavain Lake, AK	Hope et al. 2013	*M. p. drummondii*
KC473486	West of Stewart Crossing, Yukon Territory	Hope et al. 2013	*M. p. tananaensis*
KC473474	SW of Eagle, AK	Hope et al. 2013	*M. p. tananaensis*
KC473475	SW of Eagle, AK	Hope et al. 2013	

Table 2. Cytochrome *b* between group distance percentage values calculated using MEGA (Kumar et al. 2004) with a Kimura two-parameter DNA-substitution model. RI includes BIMV and MMV haplotypes. Townsend's Vole (*M. townsendii*) and Montane Vole (*M. montanus*).

Between group distance	RI	*M. townsendii*	*M. montanus*
RI		0.001	0.008
M. townsendii	0.084		0.01
M. montanus	0.068	0.078	