



Contrasting levels of genetic differentiation among populations of wolverines (*Gulo gulo*) from northern Canada revealed by nuclear and mitochondrial loci

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Abstract

Habitat loss, fragmentation, overharvest, and other anthropogenic factors have resulted in population and distribution declines for North American wolverines (*Gulo gulo*). Currently, wolverines east of the Hudson Bay are endangered and possibly extinct, whereas the status of wolverines throughout the remaining Holarctic is vulnerable. Three previous studies using nuclear loci have detected little to no significant structuring among wolverines sampled across northern Canada. Based on these results it has been suggested that wolverines in northern Canada represent a single, panmictic population. However, as has been shown in numerous studies, in cases of female site fidelity, it is possible to have demographically autonomous populations even with male-biased gene flow. To better assess the genetic structure of wolverines in northern Canada, we examined nine microsatellite loci and DNA sequence variation from a 200 bp fragment of the mitochondrial (mtDNA) control region for 270 wolverines from nine collecting areas representing three regions of northern Canada. In agreement with previous studies, microsatellite analyses revealed a lack of significant population substructure ($F_{ST} = 0.0004$). However, analysis of molecular variance, comparisons of pairwise F_{ST} values and nested-clade analysis of the mtDNA data revealed considerable genetic structuring among samples of wolverines from these three regions of northern Canada. These mitochondrial data provide evidence that wolverines in Canada are genetically structured due to female philopatry. The contrasting patterns of genetic differentiation based on nuclear and mitochondrial data highlight the importance of examining both nuclear and mitochondrial loci when attempting to elucidate patterns of genetic structure.

Introduction

Wolverines (*Gulo gulo*) are wide-ranging, solitary animals that occur at low population densities throughout tundra, taiga, and boreal forests of North America and Eurasia (Wilson 1982; Nowak 1991). Prior to human settlement in North America, wolverines were distributed throughout Canada and Alaska with populations reaching as far south as Arizona and New Mexico (Hash

1987). However, due to habitat loss, fragmentation, overharvest, and other anthropogenic factors associated with the arrival of human settlers, wolverines experienced severe population declines throughout much of their distribution (Wilson 1982). In North America, wolverines currently are distributed throughout western Canada and Alaska with individuals extending southward into remote areas of Washington, Oregon, California, Idaho, Montana, Wyoming, and Colorado (Banci

1994). Although wolverines historically occurred throughout Canada, today wolverines east of the Hudson Bay are listed as endangered (although possibly extinct) and west of the Hudson Bay wolverines have been granted special concern status (COSEWIC 2001).

Wolverines are highly vagile, with both males and females capable of long distance dispersal over short periods of time (Gardner 1985; Magoun 1985; Gardner et al. 1986; Copeland 1996; Vangen et al. 2001). Magoun (1985) reported a 300-km excursion by a female yearling over a five-month period and Gardner et al. (1986) observed a male moving 378-km over a seven-month period. Such dispersals do not appear to be influenced by topographical features such as mountains, lakes, or rivers. Supporting these observational data, low levels of genetic structuring have been revealed by nuclear loci (Wilson et al. 2000; Kyle and Strobeck 2001, 2002) and Kyle and Strobeck (2001) have gone as far as suggesting that wolverines in northern Canada represent a single, panmictically breeding population. In contrast to this low level of genetic structuring based on nuclear loci, the only study of North American wolverines that examined mitochondrial DNA detected a high degree of genetic differentiation among collecting localities ($\phi_{ST} = 0.536$; Wilson et al. 2000). Based on results of their allozyme and mtDNA data, Wilson et al. (2000) suggested that gene flow was male biased and as a result of female philopatry wolverine populations in northern Canada were structured genetically.

The need to re-evaluate the metapopulation dynamics of wolverines in northern Canada becomes more critical in light of the discordant conclusions regarding metapopulation dynamics and resulting management strategy for wolverines in northern Canada (Wilson et al. 2000; Kyle and Strobeck 2001). These discordant conclusions may, in part, be due to serious limitations of each study. At the time of publication, the study by Wilson et al. (2000) represented the largest sampling of North American wolverines, however, it was limited to 43 individuals from five localities. In contrast, the study by Kyle and Strobeck (2001) was limited by only examining biparentally inherited markers for a species that exhibits male-biased dispersal (Wilson et al. 2000) and it has been shown that at least two independent loci, preferably representing both nuclear and mitochondrial

genomes should be used when evaluating dispersal (Slatkin 1985; Avise 2000). Therefore, the objective of this study was to provide a stronger assessment of the genetic structure of populations of wolverines in northern Canada by increasing the geographical sampling and using independent loci from two genomes (microsatellites from the nuclear genome and mtDNA control region sequences).

Materials and Methods

Tissue samples from 270 trapped wolverines were obtained from nine areas in the Northwest Territories, Nunavut, and Manitoba, Canada by personnel from the Department of Sustainable Development and the Manitoba Trappers Association. Forty-three of these individuals were previously used in the mtDNA/allozyme study of Wilson et al. (2000). To avoid local effects and because of small sample sizes for some collecting localities, all samples were assigned to one of three regions (north, north-central, prairie), of which the north and prairie regions correspond with the geographical regions used by Rueness et al. (2003) for their study on Canadian lynx (*Lynx canadensis*). Tissue samples from the Northwest Territories and Nunavut were obtained from wolverine carcasses and stored at -70°C whereas desiccated skin samples were obtained from Manitoba wolverines.

Whole genomic DNA was extracted using the method of Longmire et al. (1997) and nine microsatellite loci were amplified *via* the polymerase chain reaction (PCR) with previously developed primers from wolverine (Gg-3, Gg-4, Gg-7, Gg-14, Davis and Strobeck 1998; Ggu101B, Ggu216, Ggu238, Duffy et al. 1998) and American mink (Mvi57, O'Connell et al. 1996). PCR amplification of microsatellite loci were conducted in 15 μl volumes containing 50 ng of genomic DNA, 10 pmols of each primer, 3.8 μl ddH₂O, 9 μl ABI Prism True Allele™ Premix (Perkin-Elmer Applied Biosystems, Foster City, California). The thermal profile consisted of a denaturation and enzyme activation cycle at 95°C for 12 min; 10 cycles of 94°C for 15 s, 55°C for 60 s, and 72°C for 30 s; followed by 25 cycles of 89°C for 15 s; 55°C for 60 s, and 72°C for 30 s. A final 72°C incubation for 30 min was used to ensure

that all reactions went to completion. Microsatellite variation was visualized using a Perkin–Elmer Applied Biosystem 377 Automated DNA Sequencer. GENESCAN ANALYSIS 2.02 and GENOTYPER 2.0 (Applied Biosystems, Inc., Foster City, California) software were used to determine individual genotypes.

Approximately 200 bp of the mtDNA control region were amplified by PCR using flanking primers (OSU 7863L, 5-TAAGACTCAAGG AAGAAGCAACAGC-3' AND OSU 7864H, 5'-AGCTCGTGATCTAAGTGAGA-3'). Amplifications were conducted in 50 μ l volumes containing 200–500 ng of DNA, 1 mM each primer, 1 mM Mg free buffer, 1 mM $MgCl_2$, 1 mM dNTPs, and 1 U *Taq* DNA polymerase. Cycling conditions consisted of a denaturation cycle at 94 °C for 3 min followed by 35 cycles of 94 °C for 40 s, 52 °C for 60 s, and 72 °C for 90 s. To ensure that all reactions had gone to completion, a final extension of 72 °C for 30 min was used.

Double-stranded amplicons were electrophoresed through a 0.8% agarose gel stained with ethidium bromide and exposed to ultraviolet light for visualization. Successful amplicons were purified using the Wizard PCR Prep DNA Purification System (Promega Corporation, Madison, Wisconsin) and both strands of the amplified products were sequenced using the aforementioned flanking primers and cycle sequencing according to the manufacturer's instructions (Big-Dye™ chain terminators, Applied Biosystems, Inc., Foster City, California). Cycling conditions were as follows: 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Sequence products were electrophoresed on a 377 Automated DNA Sequencer (Applied Biosystems, Inc., Foster City, California).

To test for significant departures from Hardy–Weinberg equilibrium, all nine microsatellite loci were assessed by the computer program Arlequin version 2.00 (Schneider et al. 2000). Genetic variation in terms of observed heterozygosity (H_O), unbiased expected heterozygosity (H_E), and degree of genetic structure (F -statistics; Wright 1965) were estimated by Arlequin. As an independent measure of the partitioning of genetic variation among regions, the program STRUCTURE (version 2) was used to cluster individuals into subpopulations and to ultimately reveal patterns of gene flow across northern Canada (Pritchard et al. 2000; <http://pritch.bsd.uchicago.edu>). The first

step of this analysis involved estimating the number of subpopulations (K). Three independent runs of $K = 1–9$ were performed at 200,000 MCMC repetitions and 20,000 burn-in period using no prior information and assuming correlated allele frequencies and admixture. The posterior probability was then calculated for each value of K using the estimated log-likelihood of K to choose the optimal K . In the second step of the analysis, individuals were assigned to each of the K subpopulations based upon the highest percentage of membership (q). A threshold value of $q \cdot 0.90$ was chosen (Manel et al. 2002; Cegeleski et al. 2003).

For all mtDNA sequences, the computer program AssemblyLIGN™ 1.0.9 (Oxford Molecular Group PLC 1998) was used to assemble overlapping fragments within individuals and CLUSTAL X (Thompson et al. 1997) was used to obtain a multiple sequence alignment of all individuals sequenced (the 270 individuals analyzed included the 43 individuals originally examined by Wilson et al. 2000; GenBank accession numbers AF210090–AF210132). The resulting multiple sequence alignment was subsequently imported into the computer program MacClade (Madison and Madison 2000) to identify variable nucleotide positions and the REDUNDANT TAXA option was used to compile unique sequences for further analyses. Genetic divergence among haplotypes was estimated using the distance method of Tamura and Nei (1993) with the computer program PAUP* version 4.0 (Swofford 2001). Haplotype frequencies, haplotype diversity (h), nucleotide diversity (π), and degree of genetic structure (F_{ST}) were calculated using Arlequin (Schneider et al. 2000).

The computer program TCS version 1.13 (Clement et al. 2000) was used to generate an unrooted haplotype network with resulting ambiguities in the network resolved following the recommendations of Crandall and Templeton (1993) and Templeton and Sing (1993). The haplotype network was converted into a series of nested clades: haplotypes were grouped into one-step clades, one-step clades into two-step clades, two-step clades into three-step clades until all subclades were grouped into a single clade (Templeton et al. 1987). This information, along with geographic distances between regions, was incorporated into the computer program GeoDis version 2.0 (Posada et al. 2000). GeoDis calculates

clade distance (D_c), which measures the geographic distribution of a particular clade and nested clade distance (D_n), which measures how a clade is distributed geographically in relation to its closest evolutionary sister clade (Templeton et al. 1995). The distance distribution of the nested clade versus geographical position were recalculated using 10,000 permutations under the null hypothesis of no geographic association between haplotypes. The resulting distance values of the nested design were used with a revised key (http://bioag.byu.edu/zoology/crandall_lab/programs.htm) to infer contemporary and historical factors responsible for clades showing significant associations of haplotype distribution and geography.

Results

Based on genotyping 270 wolverines for nine microsatellite loci, mean observed (H_O) and expected (H_E) heterozygosities were 0.5864, 0.4812; 0.5649, 0.6330; 0.5211, 0.6888 for the north, north-central,

and prairie regions, respectively (Figure 1). Hierarchical analysis of genetic variation revealed no significant genetic structuring among regions ($F_{ST} = 0.0004$) and this lack of genetic structuring was also revealed when regions were compared in a pairwise manner (Table 1). Results of the STRUCTURE analysis substantiated the lack of genetic structure, producing a K -value of 1.

Table 1. Pairwise comparison of genetic differentiation among the three regions in northern Canada

	North	North-Central	Prairie
North	—	0.2455*	0.2034*
North-Central	0.0010	—	0.2867*
Prairie	0.0011	0.0001	—

Values above diagonal are F_{ST} -values based on DNA sequence variation of a portion of the mitochondrial genome. Values below the diagonal are F_{ST} -values based on the nuclear microsatellite data. Values denoted by an asterisk (*) are significantly different from 0 ($P < 0.001$).

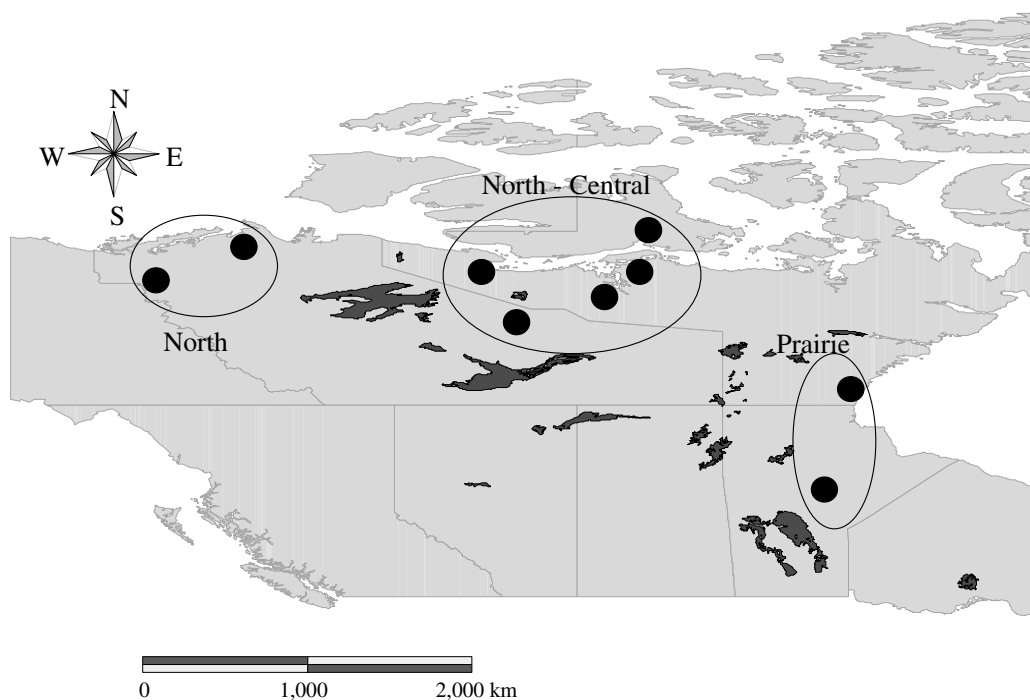


Figure 1. Map depicting sampling localities, represented by dots. Individual samples of wolverines ($n = 270$) were divided into three large-scale regions, as indicated by open circles on the map. These were north (Northwest Territories) $n = 15$, north-central (Nunavut) $n = 225$, and prairie (Manitoba) $n = 30$. Samples were taken from widespread localities and collected at “check-stations” within each region, so these do not necessarily represent populations.

An approximately 200-bp fragment of the mtDNA control region was examined for all 270 individuals. Alignment of these sequences revealed nine variable nucleotide positions (six transition substitutions, one transversion substitution, and two insertion/deletion events), resulting in 11 haplotypes (Table 2). Due to the larger sample size and increased geographic scope, we detected two haplotypes (J and K) not identified by Wilson et al. (2000). These two haplotypes were present only in samples from the prairie region. A representative sequence of haplotypes J and K have been deposited in GenBank (accession numbers AY185167 and AY185168). Percent sequence divergence among haplotypes ranged from 0.00 (two haplotypes differing by a single insertion/deletion event) to 2.97%, with a mean of 1.10%.

The number of haplotypes per region ranged from four (prairie region) to six (north and north-central regions). Within region haplotype diversity (h) was generally high whereas within region nucleotide diversity (π) was low (Table 2). In contrast to the results based on nuclear loci, hierarchical analysis of mtDNA variation revealed that 72.96% of the genetic variation was attributable to differences among individuals within regions whereas 27.04% of the variation was partitioned among regions ($F_{ST} = 0.2704$). When analyzed in a pairwise fashion, all comparisons revealed statistically significant F_{ST} values with the largest degree of differentiation being between the north-central and prairie regions ($P = 0.001$; Table 1).

Using the computer package TCS, mtDNA haplotypes separated by up to two mutational steps have a ≥ 0.95 probability of being connected in a parsimonious fashion. Using the rules of Templeton and Sing (1993), a nested design was generated. Within this network, two loops indicate ambiguous haplotype connections. The first loop contained haplotypes A, B, E, and F, whereas the

second loop contained haplotypes C, F, G, H, and J (Figure 2). Despite these ambiguities, the logic of Crandall and Templeton (1993) allowed these loops to be resolved. Although the nested contingency analyses did not incorporate geographic distance between regions, it revealed significant associations among haplotypes and their geographic locations for clades 1–3, 1–4, 2–2, and the total cladogram (Table 3). No other tests were significant at the 5% level.

The null hypothesis of no association between geographic distribution of haplotypes and mtDNA genealogy was rejected for four clades (Figure 3; Table 4). Clade 1–3 contains haplotypes C, G, and I with haplotype C being present in both the north-central and prairie

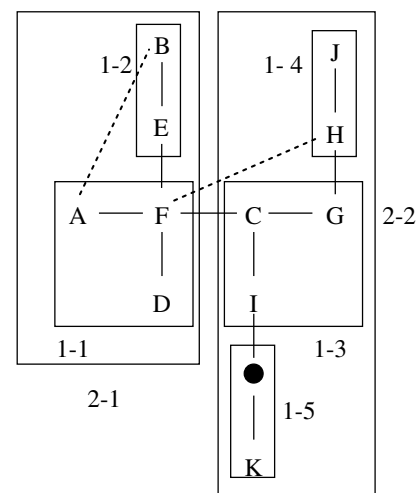


Figure 2. Unrooted estimated 95% parsimony cladogram of 11 haplotypes detected in wolverines from northern Canada. Each line in the network represents a single mutational event. Haplotypes are represented by letters A–K whereas the filled circle represents an intermediate haplotype that was not present in the sample but is necessary to link all observed haplotypes via a mutational step. Dashed lines indicate ambiguous loops.

Table 2. Distribution of each of the 11 mtDNA haplotypes among the three geographic regions depicted in Figure 1 and within region haplotype (h) and nucleotide (π) diversity along with their respective standard error (SE)

Region	mtDNA haplotypes											h	SE	π	SE
	A	C	G	H	I	F	D	B	E	J	K				
North	7				2	1	1	3	1			0.762	0.096	0.0077	0.0055
North-Central	3	46	101	6	26	43						0.709	0.020	0.0055	0.0040
Prairie	6	2								14	8	0.690	0.053	0.0153	0.0091

Table 3. Nested contingency analysis of geographic associations based on 10,000 iterations. Clades are the same as in Figure 2 and include only those clades with a probability values less than 0.05, indicating significant geographic structuring

Clade	Observed χ^2 Statistic	Probability
1-3	16.157	0.0039
1-4	20.000	0.0001
2-2	145.264	0.0000
Entire Cladogram	34.045	0.0000

regions, but having much higher frequency (95.8%) in the north-central region (Table 2). Based on our cladogram, haplotype G is derived from haplotype C and was detected only in individuals from the north-central region (Table 2). Finally, haplotype I was also derived from haplotype C and is present in individuals from the north-central regions as well as the north region but similar to the distribution of haplotype C, haplotype I is much more frequent (92.9%) in the north-central region (Table 2). Following the inference key, the most likely explanation for the distribution of these three haplotypes is restricted gene flow with isolation by distance (Table 4).

Clade 1-4 contains haplotype H, that was present only in the north-central region and haplotype J, which was detected only in individuals from the prairie region (Table 2). Although the null hypothesis was rejected for this clade, our geographic sampling was inadequate to provide an explanation for the statistically significant association of haplotypes and geography (Table 4). The explanation for the distribution of haplotypes comprising Clade 2-2, which contains clades 1-3, 1-5, and 1-5, is contiguous range expansion (Table 4). Finally, the null hypothesis was also rejected for the entire cladogram however, due to this clade being comprised of only two sub-clades, it is not possible to determine the most likely cause for the significant association between haplotype distribution and geography (Table 4).

Discussion

Three previous studies examined levels of genetic structuring among populations of wolverines from Canada based on nuclear loci (Wilson et al. 2000; Kyle and Strobeck 2001, 2002) and concluded that wolverines in northern Canada exhibit little to no geographic structuring. Our analysis of nine variable microsatellite loci from wolverines sampled

1-step clades	D	F	A	E	B	I	C	G	H	J	K
D_C	0.0	0.0	1265.2			202.4	192.1	0.0 ^S	0.0	0.0 ^S	
D_N	875.8	875.8	1219.3			88.6	86.8	33.5 ^S	1081.0 ^L	611.5 ^S	
I - T_C		-1190.8					148.2		0.0		
I - T_N		-323.3					41.3		469.4 ^L		
2-step clades											
D_C		0.0		1245.5			90.8 ^S		781.1 ^L		0.0
D_N		763.5		1152.2			303.5 ^S		866.6 ^L		1158.0 ^L
I - T_C			-1245.5						-467.2 ^S		
I - T_N			-388.9						-646.4 ^S		
Entire Cladogram											
D_C			668.2 ^L						458.5 ^S		
D_N			458.1 ^L						442.0 ^S		

Figure 3. Results of nested-clade analysis of geographical distance for wolverine mtDNA haplotypes. Haplotype designations are at the top of the figure and organized to depict the nested design shown in Figure 2. D_C and D_N are the clade distance and nested-clade distance, respectively, whereas, (I- T_C) and (I- T_N) are distances for the difference between interior and tip clades, respectively. A superscript S indicates that the measured distance was significantly small at the 5% level whereas, a superscript L indicates that the measured distance was significantly large at the 5% level.

Table 4. Interpretation of the evolutionary processes responsible for the distribution of haplotype for the clades depicted in Figure 2 for which the null hypothesis of no association between haplotype distribution and geography was rejected

Clade	Chain of inference	Final inference
1–3	1–2–3–4–no	Restricted gene flow with isolation by distance
1–4	1–19–20–no	Inadequate geographic sampling
2–2	1–2–11–12–no	Contiguous range expansion
Entire Cladogram	1–2–no	Inconclusive outcome

Final inferences were determined by use of an inference key (http://bioag.byu.edu/zoology/crandall_lab/programs.htm).

across northern Canada, failed to detect significant geographic structuring and, at least based on nuclear loci, cannot refute the suggestion that wolverines in northern Canada appear to be one large, panmictically breeding population (Kyle and Strobeck 2001). However, estimates of genetic structuring based solely on nuclear loci may be misleading and results of our mitochondrial DNA analysis highlights this point (Avice 2000). In contrast to results based on nuclear microsatellite variation, significant genetic structuring among regions was detected based on a mitochondrial locus ($F_{ST} = 0.2704$). Furthermore, all population pairwise F_{ST} values were highly significant (Table 1) indicating considerable matrilineal structuring among regions.

As an independent measure of matrilineal structuring and an attempt to elucidate the processes that have given rise to the observed patterns of haplotype distribution, we performed a nested-clade analysis. Nested-clade analysis has been shown to have more statistical power for detecting population subdivision than traditional F -statistics and may aid in distinguishing between contemporary and historic processes responsible for the observed patterns of genetic variation (Templeton 1998). Eight clades were detected in the mtDNA haplotype network for wolverines (Figure 2) and the null hypothesis of no association between haplotype distribution and geography was rejected for four of these clades (Table 4). Rejection of the null hypothesis for clade 1–3, which contains haplotypes C (found in nearly equal frequency in the north–central and prairie regions), G (detected only in individuals from the north–central region), and I (being detected in both the north and north–central regions), indicates that the most likely explanation for the geographic distribution of these haplotypes is

restricted gene flow with isolation by distance (Figure 3; Table 4) supporting significant differentiation between these two regions based on pairwise F -statistics (Table 1). Further support for differentiation between the north–central and prairie regions, as detected by pairwise F -statistics, is the rejection of the null hypothesis for clade 1–4. This clade contains haplotype H, which is restricted to the north–central region and haplotype J, which was detected only in individuals from the prairie region. Although the null hypothesis was rejected for this clade, because we did not have samples of wolverines from the area intermediate between the distribution of these two clades, we were not able to ascertain the processes that may have contributed to this pattern. However, when results of this clade are interpreted in light of the highly significant pairwise F_{ST} between these two regions, it is apparent that there is limited matrilineal gene flow occurring.

Finally, the null hypothesis was also rejected for the entire cladogram. However, due to this clade being comprised of only two sub-clades, it is not possible to determine the most likely cause for the significant association between haplotype distribution and geography (Table 4). However, examination of the haplotypes present in clades 2–1 and 2–2 suggests partitioning of mtDNA genetic variation across this landscape (Table 2). Clade 2–1 is comprised of haplotypes A, B, D, E, and F. Haplotypes B, D, and E are present only in individuals from the north region; haplotype F was detected in 43 individuals from the north–central region and a single individual from the north region (Table 2). In contrast, haplotype A, the most geographically widespread haplotype was detected in individuals from all three regions with approximately equal frequency in the north and prairie regions but lower frequency in the

north-central region. Moreover, with the exception of haplotype I which was detected only in two individuals from the north region, the geographic distribution of all other haplotypes in clade 2-2 are restricted to the north-central and/or prairie regions (Table 2). Thus, although this study supports the conclusions of previous genetic studies that have examined nuclear loci and suggested little to no geographic structuring of genetic variation in wolverines from northern Canada, the mitochondrial data clearly depicts a pattern of strong differentiation. Based on traditional *F*-statistics, all three regions are strongly differentiated from each other and the nested-clade analysis supports such differentiation.

Due to their circumpolar distribution and ability to disperse long distances in relatively short time, it has been suggested that most populations of wolverines in the Holarctic region may represent a single population. However, comparisons of levels of genetic variability from our study with results from Scandinavian wolverines indicate that North American wolverines exhibit greater levels of genetic variability than their Scandinavian counterparts. Walker et al. (2001) examined microsatellite and mtDNA sequence variation of 169 wolverines from Scandinavia and revealed expected heterozygosity values of 0.3930, 0.3450, 0.3710, and 0.3480 and are lower than detected in Canadian populations of wolverines. Sequence analysis of a 338-bp fragment of the mtDNA control region revealed only a single haplotype among all individuals whereas the number of haplotypes occurring per region in our study ranged from four to six. This single Scandinavian haplotype was not detected among the 270 individuals we examined. Moreover, the single haplotype from Scandinavia was three mutational steps different from the most closely related Canadian haplotype (haplotype H). The number of nucleotide differences between the Scandinavian haplotype and haplotypes detected in our study ranged from three to eight, with a mean of five. These nuclear and mitochondrial data indicate a long separation between Eurasian and North American wolverines and are discordant with the hypothesis that wolverines in the Holarctic region represent a single, randomly mating population.

Although a limitation of our study was the lumping of samples from several collecting locali-

ties into broad geographic regions, our analyses were able to document female site fidelity and most importantly, provide evidence that these three regions may be demographically autonomous (Avice 1995). However, it remains unclear if further subdivisions occur within each region. Therefore, additional ecological, behavioral, and genetic studies need to be conducted to better understand metapopulation dynamics of wolverines in North America. Future genetic studies should attempt to include additional individuals from the regions sampled in this study, intermediate areas between our three regions, and between regions sampled by Kyle and Strobeck (2001, 2002).

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