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# Characterizing the elusive Vancouver Island wolverine, *Gulo gulo vancouverensis*, using historical DNA

Evan W. Hessels, Eric C. Lofroth, Richard D. Weir, and Jamieson C. Gorrell*

*Biology Department, Vancouver Island University, Nanaimo, BC V9R 5S5, Canada (EWH, JCG)*
*Boreas Ecological, Victoria, BC V9E 2B1, Canada (ECL)*
*Ministry of Environment, Government of British Columbia, Victoria, BC V8W 9M1, Canada (RDW)*

**Correspondent: Jamie.Gorrell@viu.ca*

The wolverine (*Gulo gulo*) is a Holarctic species found in North America primarily across the boreal forest, the subarctic, and along the Pacific coast, including Vancouver Island (VI), British Columbia. While wolverines on VI are rare and possibly extirpated, they have been previously described as a unique subspecies, *G. g. vancouverensis*, distinct from *G. g. luscus* from the mainland of North America. However, the validity of the VI subspecies is contentious, with conflicting results from studies of skull morphology. Here, we used molecular analyses to characterize the genetic diversity of the VI population and resolve this taxonomic debate to assist with conservation priorities. Historical DNA of VI wolverines was obtained from museum specimens, amplified at 16 nuclear microsatellite loci, and sequenced at the mitochondrial D-loop control region to compare with wolverines from mainland British Columbia. The VI population had lower allelic richness and was fixed for a single common mtDNA haplotype. Bayesian and non-Bayesian assignments using microsatellites generally revealed admixture across populations, implying allele frequencies between the VI and mainland populations were not significantly different. Hence, both types of genetic markers showed little evolutionary divergence between VI and the mainland population. Combined, these results do not provide evidence of significant genetic distinction for VI wolverines, nor support the subspecific classification. Immediate conservation efforts should focus on estimating population size, while future conservation planning can assume VI wolverines likely are not a unique genetic population and there remains the potential for natural recolonization of wolverines to VI.

Key words: *Gulo gulo*, historical DNA, microsatellite, mtDNA, museum specimen, subspecies, Vancouver Island, wolverine

Speciation is known to occur by means of numerous different processes. One of the most well-documented is through the physical isolation of individuals from their ancestral population. Over time, isolation can lead to significant genetic differentiation via selective forces and neutral processes (i.e., founder effects and genetic drift). In concert, these factors often are the drivers of allopatric speciation (Barton and Charlesworth 1984). Geographic barriers are the most common cause of isolation: these are any physical barriers that prevent populations from interacting (Zimmer and Emlen 2015). Allopatric speciation due to isolation in conjunction with in situ speciation is particularly important in explaining the unique diversity found on island habitats around the world (Presgraves and Glor 2010; Matzke 2014; Eldridge et al. 2018; Heaney et al. 2018).

During the Pleistocene (last glacial maximum), Vancouver Island (VI), British Columbia, Canada maintained ice-free regions that provided glacial refugia for a variety of species (Hebda and Haggarty 1997; Walser et al. 2005; Godbout et al. 2008; Shafer et al. 2010). As global temperatures warmed and glaciers receded, land bridges were lost and previously passable ice sheets became impassable (Chavez et al. 2014). Consequently, island populations became isolated and gained the potential to diverge from their mainland counterparts (Zimmer and Emlen 2015). Approximately 20,000 years ago, two species of North American tree squirrel, *Tamiasciurus hudsonicus* and *T. douglasii*, became isolated in a refugium on VI. Once gene flow to the mainland no longer occurred, both species began hybridizing until *T. douglasii* was lost, leaving *T. hudsonicus* with evidence of introgression (Chavez et al. 2014).

The hoary marmot (*Marmota caligata*) is believed to have undergone rapid speciation on VI during the last glacial maximum, becoming a distinct species, the Vancouver Island marmot (*Marmota vancouverensis*; Cardini et al. 2007).

However, some populations experiencing either complete or partial isolation from their ancestral population might not have been isolated long enough to accumulate sufficient differences to be identified as distinct species. Instead, these populations may be diagnosably distinct subspecies appearing to be separately evolving lineages with discontinuities resulting from restricted gene flow (e.g., geography, ecological specialization—Taylor et al. 2017). The description of a subspecies is highly debated as multiple definitions exist ranging from geographic discontinuities in morphological traits to delineating phylogenetic clades (Patton and Conroy 2017; Ruedas 2020). The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) defines subspecies based on the identification of Designatable Units (DUs). DUs consider a variety of population attributes which are roughly split into the overall discreteness of the population, as well as its evolutionary significance. The discreteness of a population is based on several criteria including evidence of genetic distinctiveness, natural isolation from other regions (e.g., islands), as well as occupation of unique ecogeographic regions that may reflect historical or genetic distinction (COSEWIC 2015). Distinctiveness is determined largely using both physical appearance, including coloration and skeletal morphology (Patton and Conroy 2017), as well as variation across nuclear and mitochondrial genomes (Selkoe and Toonen 2006). COSEWIC (2015) recommends measuring the evolutionary significance of a population relative to other populations using the following criteria: 1) differences in characteristics that may represent relatively deep intraspecific phylogenetic divergence (including fixation at functional genes, stable cultural behavior, and qualitative differences in slow evolving genetic markers); 2) the persistence of a population in a unique or unusual ecological setting in which local adaptations are likely to, or have developed; 3) the discrete population is the only surviving natural occurrence of its species, with a more abundant introduced population outside of its historical range; or 4) if loss of the population will cause disjunction affecting the natural dispersal of the species. Once a population is assessed as a DU, it may be considered a separate subspecies (COSEWIC 2015).

Before the development of molecular methods, morphological techniques were the primary method of determining differences among populations. Characterization of morphological differences generally is a simpler and less expensive method to distinguish taxonomic ranks compared to molecular methods because it does not always require specialized equipment (Stein et al. 2014). Molecular analyses allow for nonlethal sample collection and can typically determine differences among populations that otherwise would not be exposed by morphological analyses (Arbogast et al. 2017; Rosel et al. 2017). While both methods can be effective, they do not always yield the same results (Laurence et al. 2011; Arbogast et al. 2017). This could reflect phenotypic plasticity, where morphological variation may in part be caused by environmental factors (Nicolas et al. 2010) or it also may be the product of sequencing regions of the genome that are not responsible for specific morphological variation (Patton and Conroy 2017). At present, a more common method of resolving taxonomic distinction is to include genetic and morphological analyses (Dayrat 2005; Padial et al. 2010; Schlick-Steiner et al. 2010; Solari et al. 2019).

Subspecies classifications are being challenged constantly and reevaluated as more information becomes available. In many cases, this is due to the development of new techniques that can help resolve taxonomic debates (Rosel et al. 2017). For example, some morphologically defined subspecies of muskrat (*Ondatra zibethicus*) could not be differentiated from each other through genetic analyses (Laurence et al. 2011). In contrast, some morphologically defined subspecies of flying squirrel (*Glaucomys sabrinus*) recently were elevated to species as a result of genetic analyses (Arbogast et al. 2017). However, morphological and genetic analyses can be in agreement, as demonstrated by the recent discovery of a new subspecies of mouse (*Mus musculus helgolandicus*) on the island of Heligoland, where a single colonization event hypothesized to have taken place 400 years ago led to skeletal and genomic distinction in a short span of time (Babiker and Tautz 2015). These reassignments of taxonomic rank can alter the perceived distributions and abundances of populations and species. Despite limited available resources, the reclassifications can then prompt conservation actions (e.g., threatened or endangered—Mace 2004; COSEWIC 2015). For example, variation in floral morphology split *Pterostylis* orchids into 26 genera, while subsequent genetic analyses lumped them back together again under a single genus, alleviating immediate conservation concerns (Janes et al. 2010, 2012; Janes and Duretto 2010).

Wolverines (*Gulo gulo*) occur throughout much of the Holarctic, in both the Western and Eastern Hemispheres. Two distinct subspecies are recognized globally: *G. g. gulo* in the Eastern Hemisphere across Eurasia, and *G. g. luscus* in the Western Hemisphere, believed to have migrated from a single Beringial refugium across North America following the last glacial retreat (Pasitschniak-Arts and Larivière 1995; Zigouris et al. 2013). On the western shore of North America, British Columbian and Washington wolverine populations mostly occur in forested areas of the western mountains, away from the coast (Aubry et al. 2007; COSEWIC 2014), although there have been rare individual sightings on beaches. Wolverines are abundant throughout the northern two-thirds of British Columbia, but are much less common in the southwestern corner of the province and on VI (Lofroth and Krebs 2007; Hatler et al. 2008). However, wolverines are known to historically have occupied VI and have been spotted on Pitt and Princess Royal islands (MacLeod 1950; Shardlow 2013) off the coast of British Columbia, north of VI. Wolverines in British Columbia are listed as a species of special concern by COSEWIC, with additional concern regarding the southern areas of the province (COSEWIC 2014). In Washington, only small populations of wolverines remain that are protected under state listing and given a critically imperiled designation by NatureServe (COSEWIC 2014).

After examining two skulls, Goldman (1935) determined that wolverines from VI differed sufficiently from those of the mainland to be classified as a third subspecies, *G. g. vancouverensis*. However, Banci (1982) reevaluated the VI population with seven additional samples and concluded that, despite differences in cranial dimensions, these differences were not large enough to classify *G. g. vancouverensis* as a unique subspecies. Despite this evaluation, Banci (1982) nevertheless suggested that the subspecies designation could be retained for management purposes.

The VI wolverine is "red-listed" provincially as a possibly extirpated or historical population because current abundance estimates are unavailable, although likely extremely low at best (B.C. Conservation Data Centre 2019). The potential subspecific designation increases the conservation concerns over this possibly unique but elusive population. The most recent federal status report recognizes only a single DU across Canada while classifying wolverines as "s*pecial concern*," but also highlights the need for further genetic analysis of the VI population (COSEWIC 2014). In this study, we analyzed DNA from museum specimens to genetically characterize the VI wolverine population and quantify the amount of genetic differentiation between the island and mainland populations to determine if the subspecific designation of *G. g. vancouverensis* is warranted.

## Materials and Methods

*Wolverine samples and DNA extraction.*—Eight VI wolverine specimens (including six used in Banci's (1982) morphometric study) were located in Canadian and American museum collections (Table 1; Fig. 1). Various genetic samples were collected from each specimen, depending on preservation technique and available tissues. In the case of skeletons, small holes were drilled into molar roots or bone to extract genetic material, and dried flesh was sampled when available. For preserved pelts, tissue was extracted from the nail beds similarly by drilling, as this region is less affected by the tanning process, which can severely degrade DNA (Merheb et al. 2015). DNA was extracted from all samples using a DNeasy Blood and Tissue kit (Qiagen, Toronto, Ontario, Canada) in a laboratory without previous wolverine research so as to avoid contamination. We followed the manufacturer's recommended procedure except for two modifications. First, tissue was incubated in 180 µl ATL buffer and 20 µl Proteinase K for 12–24 h at 56°C to allow for maximum digestion. Second, the final DNA elution step was reduced to 100 µl Buffer AE to allow for a greater concentration of DNA in the final solution.

*Mitochondrial sequence analysis.*—A 384-bp fragment of the D-loop in the mtDNA control region was amplified using polymerase chain reaction (PCR) with the universal primer H16498 (Shields and Kocher 1991) and the wolverine primer Gulo0F (Schwartz et al. 2007); negative controls were included in all PCRs. PCR protocols were based on those described by Schwartz et al. (2007) with a final reaction volume of 25 µl, composed of 12.5 µl of Top Taq PCR Master Mix (Qiagen, Toronto, Ontario, Canada), 1 µl of 10 µM primer mix, 1.2 µl of additional $MgCl_2$ (25 mM), 8.3 µl of $H_2O$, and 2 µl of genomic DNA (~10–20 ng). The PCR program was set at 94°C for 5 min, then 44 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 5 min at 72°C. PCR products were visualized in a 1% agarose gel containing SYBR Safe gel stain (ThermoFisher Inc., Waltham, Massachusetts) and 1× Tris-borate-EDTA buffer. Amplification was confirmed by comparing bands to a 2-Log DNA ladder (New England Biolabs, Ipswich, Massachusetts). The amplified DNA was sent to the Molecular Biology Service Unit (University of Alberta, Edmonton, Alberta, Canada) for Sanger sequencing using a BigDye Terminator 3.1 kit (ThermoFisher) and an ABI3730 capillary DNA analyzer. DNA fragments were sequenced using both the forward and reverse primers to clarify any uncertainty arising from primer-dimer products that may interfere with base calling. BioEdit (Hall 1999) was used to clean and review chromatographs as well as combine the forward and reverse sequences for each individual. Haplotypes were identified using nucleotide BLAST optimized for highly similar sequences (megablast), in comparison to all known wolverine haplotypes in GenBank. Sample 10678 was previously sequenced using the same primers by Zigouris et al. (2013) and

**Table 1.—**Vancouver Island wolverine specimens including archive number, museum, year of collection, sex, sampling location, and tissue type used to extract DNA. The last three columns indicate which specimens were measured by Banci (1982) and had successful DNA amplification in the current study.

| Archive # | Museum[a] | Year | Sex[b] | Location[b] | Tissue type | Banci | Amplification success | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Microsatellite | Mitochondrial |
| 1343 | RBCM | 1891 | M | n/a | Dried tissue | Yes | No | No |
| 1570 | RBCM | 1907 | M | Cowichan Lake | Nail bed, dried tissue, tooth | Yes | No | No |
| 2486 | RBCM | 1937 | M | Cameron River | Nail bed | Yes | Yes | Yes |
| 9872 | RBCM | 1978 | F | Rooney Lake | Nail bed, dried tissue | Yes | Yes | Yes |
| 1745 | UBC | 1946 | F | Tsable River | Nail bed, dried tissue, bone marrow | Yes | Yes | Yes |
| 3510 | UBC | 1949 | n/a | Cameron Lake | Molar root | Yes | No | Yes |
| 13006 | MVZ | 1910 | n/a | Friendly Cove | Hide | No | No | Yes |
| 10678 | UCM | 1910 | n/a | n/a | Tooth, turbinate bones | No | No | Yes[c] |

[a]RBCM = Royal BC Museum, Victoria; UBC = Beaty Museum, University of British Columbia, Vancouver; MVZ = Museum of Vertebrate Zoology, University of California, Berkeley; UCM = University of Colorado Museum, Boulder.
[b]n/a indicates information not available.
[c]Previously sequenced by Zigouris et al. (2013).

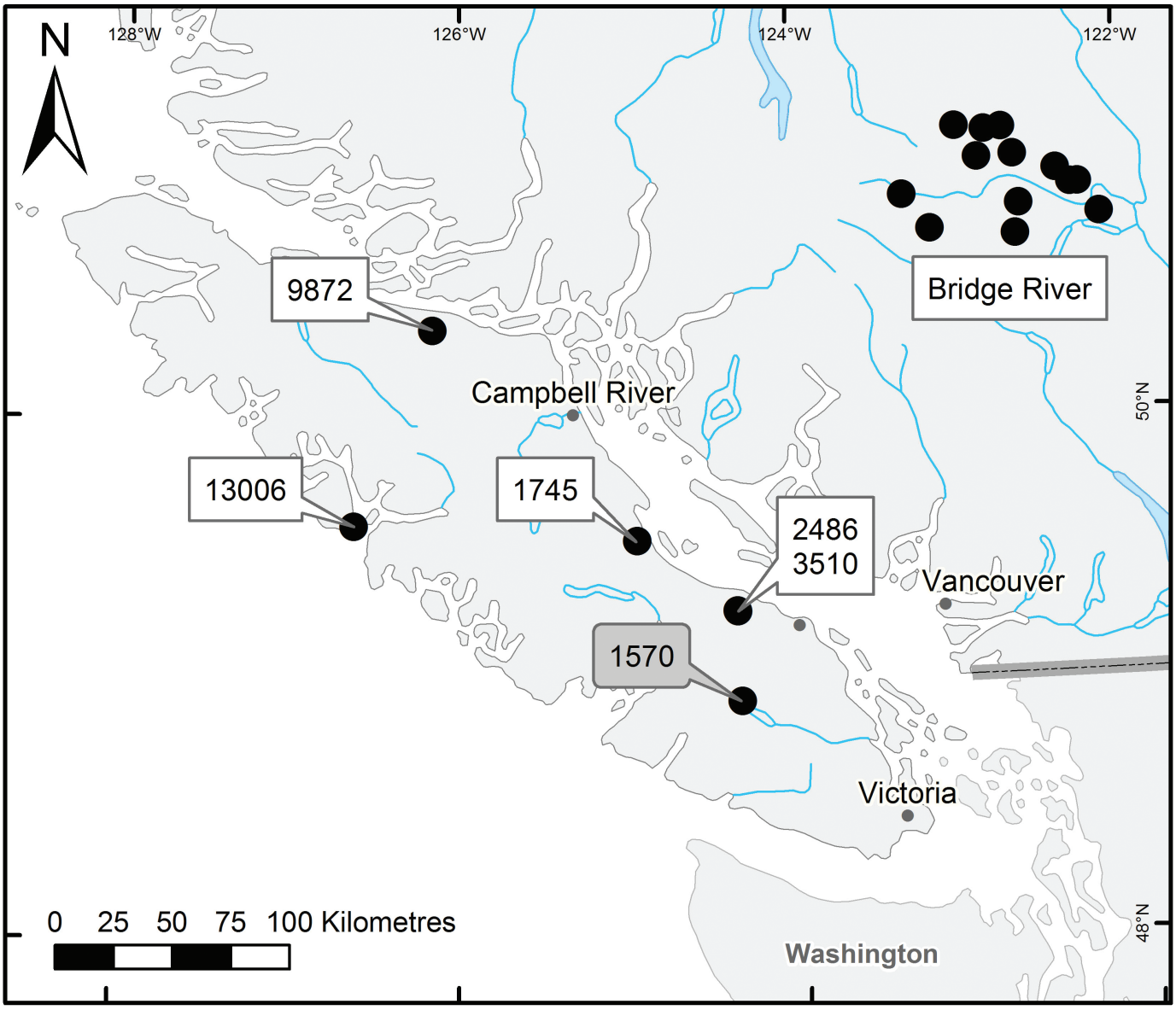


**Fig. 1.**—Location of Vancouver Island wolverine specimens including catalog numbers, with successfully amplified mtDNA samples (white) and one unsuccessful sample (gray). Two specimens (1343 and 10678) are not included as specific locations are not available. Bridge River represented the mainland population with 15 wolverines sampled at 13 locations.

was not reamplified in our laboratory, although we included the sequence from GenBank (accession number AF210090) in our analyses.

*Microsatellite analysis.*—An aliquot of DNA from each specimen was sent to Wildlife Genetics International (Nelson, British Columbia, Canada) for microsatellite genotyping across 17 loci (Supplementary Data SD1) following established protocols (Paetkau et al. 1998) to ensure consistent allele scoring with recently genotyped tissue samples from 15 wolverines sampled near Bridge River, British Columbia. (Fig. 1). Samples from Bridge River were used as our mainland population and represented *G. g. luscus* for comparative analyses, which likely is the closest mainland population to VI because wolverines are not as common in the southwestern corner of the province (Lofroth and Krebs 2007; Hatler et al. 2008). Successfully amplified samples were genotyped two or more times to ensure consistent scoring; genotyping error rate was estimated to be less than 1% based on perfect repeat scores. We tested for deviation from Hardy–Weinberg equilibrium and linkage disequilibrium while implementing a Bonferroni correction using Genepop 4.7 (Raymond and Rousset 1995). We used GenAlEx 6.5 (Peakall and Smouse 2006, 2012) to determine probability of identity, allelic diversity ($N_A$), observed heterozygosity ($H_O$), unbiased expected heterozygosity ($uH_E$), and Wright's fixation index ($F_{ST}$), with 999 permutations for statistical significance. In addition, we estimated allelic richness (rarefied allelic diversity) using HP-Rare (Kalinowski 2005) to account for uneven sampling. The data were rarefied to twice the smallest sample size corresponding to the maximum number of alleles that could possibly be identified ($n = 6$). Differences in mean allelic

richness and expected heterozygosity between the VI and mainland populations were compared with Wilcoxon Signed-Rank Tests using the Real Statistics Resource Pack for Excel (Zaiontz 2013).

Population genetic structure was assessed using a Bayesian approach in the program STRUCTURE (Pritchard et al. 2000). We examined $K = 1 - 4$ with 75,000 generations discarded as burn-in and 250,000 MCMC repetitions using correlated allele frequencies in an admixture model with 20 iterations per $K$ (cluster). Separate analyses were performed with and without sample origin as a prior (*locprior* option) to help detect any weak population structure (Hubisz et al. 2009). Because of uneven sampling between populations, we determined the optimal number of clusters with the ln Pr($X|K$) as well as the median of means (MedMeanK) and the median of medians (MedMedK) for each given $K$ (Puechmaille 2016) using StructureSelector (Li and Liu 2018), and visualized the cluster plots using CLUMPAK (Kopelman et al. 2015). We did not rely on the $\Delta K$ method (Evanno et al. 2005) due to known biases toward $K = 2$ (Cullingham et al. 2020), or re-analyze each cluster for hierarchical subdivision as recommended by Janes et al. (2017) due to a limited sample size and the relevant focus of comparing only two populations. As an alternative approach to STRUCTURE, we also carried out a discriminant analysis of principal components (DAPC) using the *adegenet* 2.1 package (Jombart and Ahmed 2011) in R 3.6 (R Core Team 2019) to determine the cluster membership probabilities among samples. We tested $K = 1$–4 clusters and determined the optimal number of clusters based on the lowest BIC value. We optimized the *a*-score and employed cross-validation (training with 90% of data, repeated 30 times) to minimize the number of principal components and avoid overparameterizing the final analysis.

On account of the possibility of substructure within the mainland population, we also carried out non-Bayesian assignment tests with the *geneplot* package (McMillan and Fewster 2017) in R to test how the VI samples would match with the mainland population. We undertook a simple assignment test using both VI and Bridge River populations as reference groups. We also used the individual cluster membership probabilities from the DAPC ($q \geq 0.79$) to split mainland samples into multiple reference groups to calibrate genotype likelihoods before assigning the VI samples. We selected the *leave-one-out* method and the prior from Baudouin and Lebrun (2001) to account for small sample sizes.

## Results

*Haplotype diversity.*—We successfully sequenced the full 384-bp fragment in five out of seven VI wolverines with all five individuals sharing the same haplotype as the VI specimen previously sequenced by Zigouris et al. (2013). This sequence was labeled as haplotype "A" by Wilson et al. (2000), Chappell et al. (2004), and Cegelski et al. (2006), as haplotype "B" by Tomasik and Cook (2005), and as haplotype "1" by Zigouris et al. (2013).

*Microsatellite genotypes.*—We successfully genotyped three of the eight VI wolverine specimens at all 17 loci, which were compared to 15 samples from the Bridge River area on mainland British Columbia. The three VI samples differed from each other at eight or more loci, providing high confidence of unique genotypes for each sample. The population-specific probabilities of identity were $2.1 \times 10^{-9}$ for Bridge River and $1.5 \times 10^{-5}$ for the three VI samples. Though the VI population did not exhibit deviation from Hardy–Weinberg at any locus, Bridge River showed significant deviation at one locus (MP0182, $P < 0.0003$) which was omitted from further analysis. We found no evidence of linkage disequilibrium for either population after Bonferroni correction.

Wright's fixation index suggested significant genetic variance between the VI and mainland populations ($F_{ST} = 0.17$, $P < 0.001$). While the two populations showed no significant difference in unbiased expected heterozygosity ($uH_E$; VI: 0.39 ± 0.07 *SE* versus Bridge River: 0.51 ± 0.05 *SE*; $z = 1.16$, $P > 0.25$), the VI population had significantly lower allelic richness ($A_R$) compared to Bridge River (1.9 ± 0.2 *SE* versus 2.4 ± 0.2 *SE*, respectively; $z = 2.15$, $P < 0.02$; Table 2). We found that the VI samples had two private alleles across two loci, whereas Bridge River had 30 alleles across 15 loci that appeared exclusive to the mainland population.

Our first STRUCTURE analysis (excluding sample origin) determined a maximum mean ln Pr($X|K$) value at $K = 2$ (Supplementary Data SD2) with VI and Bridge River showing variable levels of individual admixture to both clusters consistently across 19 of 20 runs (Fig. 2A). However, the median $K$ estimators (MedMeanK and MedMedK) only identified one genetic cluster ($K = 1$) as a more conservative interpretation. With the addition of sample origin as a prior in the second STRUCTURE analysis, the mean ln Pr($X|K$) suggested $K = 3$ although the median $K$ estimators suggested only two distinct clusters (Supplementary Data SD2). At $K = 2$, VI samples grouped together and assigned strongly to one cluster while all Bridge River samples assigned strongly to the other cluster with little admixture (Fig. 2B). These individual assignments were consistent across 14 of 20 runs, though there was considerably more admixture in the Bridge River population in the remaining six runs (Supplementary Data SD2). Finally, the DAPC revealed $K = 3$ as the optimal number of clusters according to the BIC values, and we only retained two principal components in the final analysis, to minimize the mean

**Table 2.—**Sample size ($n$), allelic diversity ($N_A$), allelic richness ($A_R$), observed heterozygosity ($H_O$), and unbiased expected heterozygosity ($uH_E$) with standard errors from 16 microsatellite loci for Vancouver Island and mainland wolverines.

| Sampling area | $n$ | $N_A$ | $A_R$ | $H_O$ | $uH_E$ |
|---|---|---|---|---|---|
| Vancouver Island, British Columbia | 3 | 1.9 ± 0.2 | 1.9 ± 0.2 | 0.35 ± 0.08 | 0.39 ± 0.07 |
| Bridge River, British Columbia | 15 | 3.7 ± 0.4 | 2.4 ± 0.2 | 0.47 ± 0.06 | 0.51 ± 0.05 |

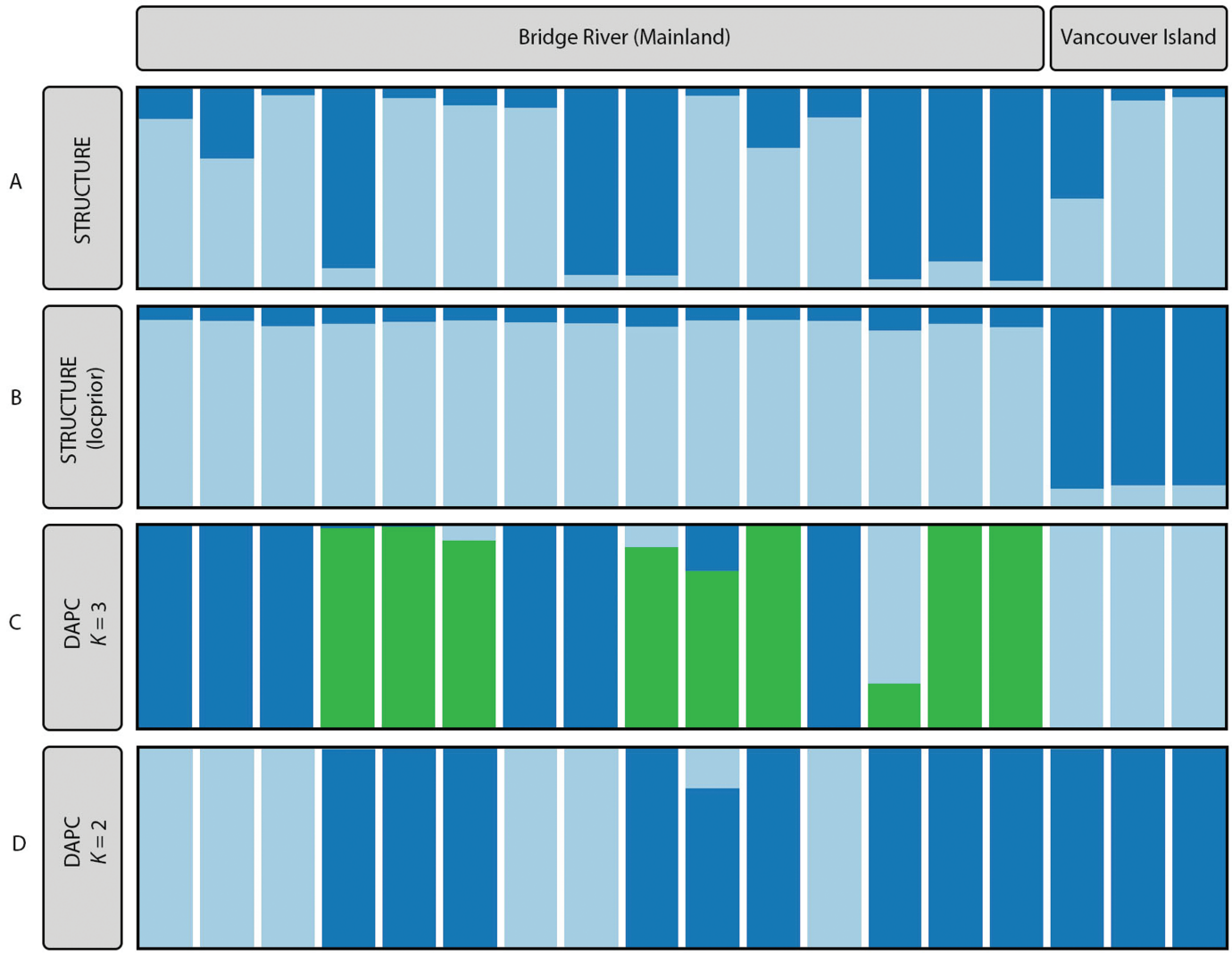


**Fig. 2.**—Membership probabilities among samples from Vancouver Island ($n = 3$) and mainland British Columbia ($n = 15$) using 16 microsatellite loci. (A) STRUCTURE found significant admixture between populations at $K = 2$ when omitting sample origin (19/20 runs) but found evidence of differentiation (14/20 runs) when including origin as a prior in the analysis (B). A discriminant analysis of principal components (DAPC) revealed admixture between populations at (C) $K = 3$ and (D) $K = 2$.

squared error (Supplementary Data SD3). These genetic clusters separated individuals into three similarly sized groups that were equally related to each other. Visualizing the membership probabilities revealed that Bridge River individuals assigned to all three clusters while all three VI samples assigned to one of these clusters (Fig. 2C). Taking a more conservative approach to avoid oversplitting one population, we also visualized $K = 2$ and found that all VI samples assigned strongly to one of the two Bridge River clusters (Fig. 2D).

The GenePlot assignment tests revealed weak differentiation between mainland and VI populations (Fig. 3). The simple assignment test with both populations included as reference groups revealed some separation between populations except that one Bridge River sample had a higher likelihood of originating from VI (Fig. 3A). Splitting Bridge River into two reference groups according to the DAPC cluster membership probabilities ($K = 2$, $q \geq 0.79$) revealed that VI samples were equally likely to assign to either cluster (Fig. 3B). Further splitting of Bridge Ridge into three reference groups failed to isolate the VI samples from the mainland samples (Supplementary Data SD3).

## Discussion

We assessed the genetic distinctiveness of wolverines from VI to determine if they are a unique subspecies or if instead they should be considered a (possibly extirpated) population of the mainland subspecies. Resolving this taxonomic classification likely will influence future wolverine conservation priorities and strategies on VI. Our results determined that VI wolverines did not appear to be differentiated substantially enough in a genetic perspective from their mainland counterparts to justify separate classifications.

*Population genetic structure.*—Mitochondrial analyses revealed that six specimens from VI all shared the same haplotype, which also is the most common haplotype across western North

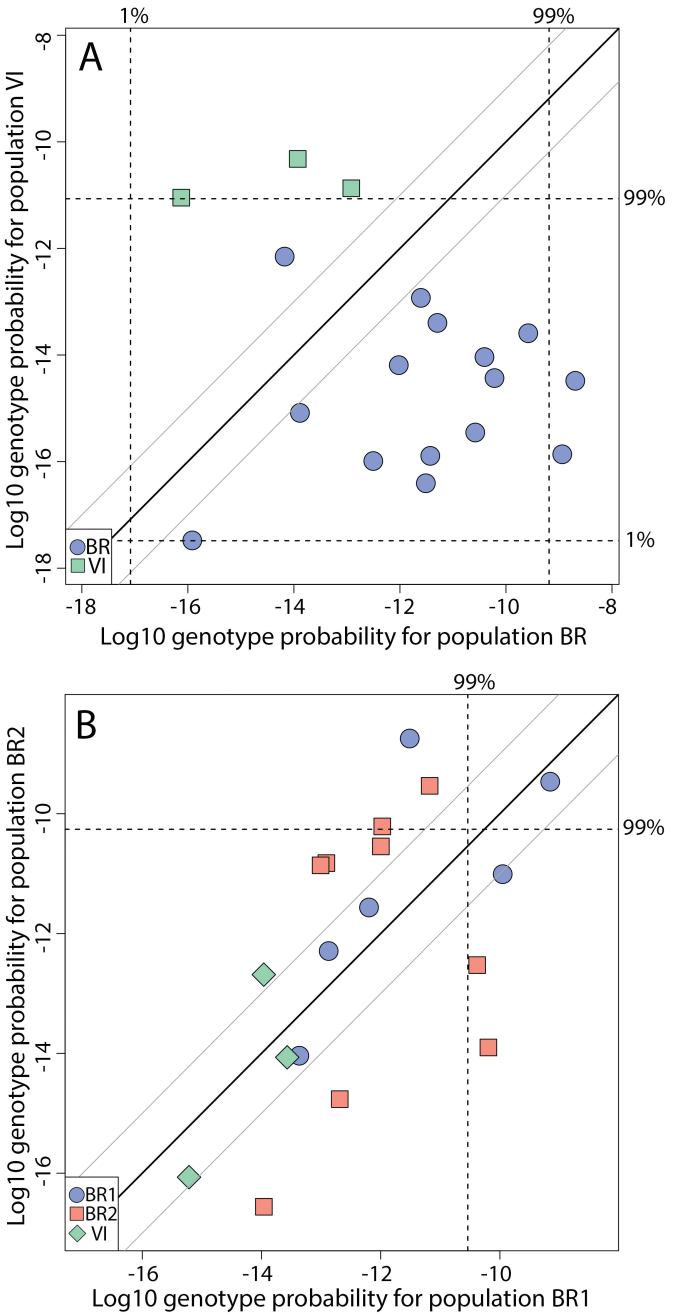


**Fig. 3.**—Assignment tests of samples from Vancouver Island ($n = 3$) and Bridge River ($n = 15$). (A) Simple assignment test with both populations included as reference groups. Note that one Bridge River (BR) sample had a higher likelihood of originating from Vancouver Island (VI). (B) Bridge River was split into two reference groups according to the discriminant analysis of principal components (DAPC) cluster membership probabilities ($K = 2$, $q \geq 0.79$) to test the assignment of VI samples.

America (Zigouris et al. 2013). Hence, the VI population does not appear to have any unique mitochondrial haplotypes, which can otherwise be good indicators of isolation and divergence of a population (Chavez et al. 2014; Arbogast et al. 2017).

The lack of diversity in VI wolverine haplotypes may be the result of historical founder effects or drift since colonization of VI after the last glacial retreat (Zigouris et al. 2013). This loss of diversity would remain fixed even with recent male-mediated gene flow (as has been suggested by Wilson et al. 2000; Chappell et al. 2004; Tomasik and Cook 2005; Cegelski et al. 2006) onto VI as mtDNA is maternally inherited. In Labrador, Canada, herds of woodland caribou (*Rangifer tarandus*) were isolated during the last glacial recession leading to genetic variation among populations (Wilkerson et al. 2018). While differentiation was attributed to multiple founder effects, the lack of haplotype diversity in one of the four populations was believed to be caused by high mortality and drift in a small population, something which likely also occurred within the VI wolverine population.

Similarly, our nuclear microsatellites failed to separate VI wolverines from the mainland consistently when using either Bayesian or non-Bayesian assignments tests. Only the second STRUCTURE analysis (including sampling origin) was able to fully separate these two populations (though inconsistently in 14 of 20 runs), while all other analyses showed varying degrees of admixture or cross-assignment. Hence, when considering the STRUCTURE results that did not include sample origin, as well as those from the DAPC and the GenePlot assignment test, we conclude that there is no more variation between the two populations than there is within the Bridge River population itself. This weak structure is no greater than that typically expected among populations separated across continuous habitat but is less than that expected for an island population isolated by an oceanic barrier, and certainly given the amount of time since VI has been isolated from the mainland (Chavez et al. 2014).

While the second STRUCTURE analysis is able to separate the populations, this alone is not enough evidence to indicate strong genetic differentiation. Including sample origin in the analysis can expose weak structure that otherwise may remain hidden; however, this structure is minor in comparison to any divergence detected when omitting origin from the analysis (Hubisz et al. 2009). Haché et al. (2017) were able to distinguish one subspecies of ovenbird (*Seiurus aurocapilla*) only when including sample origin; however, ovenbirds showed much stronger consistency of separation across replicate runs and had a considerably larger sample size. Alternatively, small sample size may exacerbate our results because the few samples from VI limited the number of alleles and haplotypes that could be detected. Although rarefaction and Bayesian analyses to some extent may correct for uneven sample sizes, we cannot rule out the possibility that our results may be influenced by low sample size and accordingly, should be interpreted conservatively (Kalinowski 2005; Hubisz et al. 2009; Hale et al. 2012).

Allelic richness was significantly lower on VI than on the mainland. Reduced allelic richness can result from small sample sizes or allelic dropout with low-quality DNA. Wandeler et al. (2003) suggested that the risk of allelic dropout increases with the length of amplified fragments when DNA is degraded. However, alleles from the four loci that were monomorphic in the VI population were the same length as those from polymorphic loci. When also considering we had perfect

repeat scores when genotyping samples two or more times, and that museum samples were unique from each other, we feel confident that our DNA was not significantly degraded enough to increase the likelihood of allelic dropout in our data. In addition, VI samples had few private alleles in comparison to those found in the mainland samples, something unexpected considering the possible uniqueness of the population.

Alternatively, lower richness and increased population subdivision also can be indicative of reduced gene flow and genetic drift, which are the necessary first steps in speciation (Barton and Charlesworth 1984; Arbogast et al. 2017). Our estimate of population subdivision based on heterozygosity ($F_{ST}$ = 0.17) was significant, but still within the range reported in most wolverine studies across North America (i.e., $F_{ST}$ = 0.00–0.22—Kyle and Strobeck 2001, 0.00–0.20—Kyle and Strobeck 2002, 0.06–0.17—Cegelski et al. 2003, 0.01–0.21—Cegelski et al. 2006, and 0.07–0.45—Schwartz et al. 2007). However, the $F_{ST}$ value in our study may be inflated due to the small sample size and uneven sampling between populations, creating biased estimates of heterozygosity and in turn $F_{ST}$ (Meirmans and Hedrick 2011). Pairwise $F_{ST}$ generally decreases with increasing sample size (Hale et al. 2012).

Comparing historical VI specimens to modern mainland samples also includes a confounding temporal aspect that may increase the amount of genetic structure observed here. Historical wolverine populations in Sweden and California both showed more differentiation from modern populations than modern populations did from each other (Walker et al. 2001; Schwartz et al. 2007). Overharvesting and small populations likely accelerated the effects of drift and increased $F_{ST}$ over time in these populations, a phenomenon that also may apply to VI wolverines.

*Subspecies consideration and conservation implications.*—The results from both the microsatellite and mitochondrial analyses presented here indicate a lack of evolutionary divergence between VI and mainland wolverines. Moritz (1994) defined evolutionary significant units (ESUs) as populations that are monophyletic for mtDNA haplotypes and exhibit significant divergence in nuclear allele frequencies. For example, wolverines in California were thought to have been isolated from the rest of North America since 1893 (Grinnell et al. 1937). Historical DNA analyses reveal that Californian wolverines had unique mtDNA haplotypes compared to Alaska, central Idaho, and greater Montana with microsatellite pairwise $F_{ST}$ estimates between 0.31 and 0.45 (Schwartz et al. 2007), which were greater than what we observed for VI specimens (0.17). Similarly, mice on the island of Heligoland were classified as a distinct subspecies after both microsatellite and mtDNA results showed significant differentiation from the mainland populations (Babiker and Tautz 2015). By comparison, while we found that VI and the mainland showed evidence of private alleles, this likely is attributable either to reduced gene flow (island effect) or low sample size. Our Bayesian results provided evidence that VI and mainland populations had only minor differences in microsatellite allele frequencies between them and no unique mitochondrial haplotypes were discovered. This combined with the results of Banci's (1982) morphometric study leads us to conclude that the VI wolverine is not significantly distinct from the mainland population and there is currently not enough morphological or genetic evidence to support the VI population as an ESU or a distinct subspecies (Coates et al. 2018).

However, before lumping North American wolverines into a single taxonomic classification, we still must consider the historical and geographic criteria that COSEWIC (2015) uses to define a subspecies. VI is a unique environment and includes endemic species that have diverged from the mainland (e.g., Cardini et al. 2007). Maintaining the subspecies classification may still be warranted if geographic isolation prevents wolverines from dispersing to VI. Some carnivores do traverse the Discovery Islands or the Johnstone Strait to reach the northeast coast of VI which can be as little as 1–3 km away from the mainland. Brown bears (*Ursus arctos*) regularly arrive on the VI shores between Campbell River and Port McNeill with some individuals choosing to stay extended periods of time before returning to the mainland. Wolves (*Canis lupus*) also have been observed swimming across, and likely recolonized VI using this passage after being virtually extirpated in the 1950s (Muñoz-Fuentes et al. 2010). This suggests that the channel is traversable and might offer a potential dispersal corridor for wolverines.

Two wolverines were recently spotted on Princess Royal Island (53°N, 129°W—Shardlow 2013) suggesting they swam across the Princess Royal Channel (approximately 1 km wide). Furthermore, we have recorded three radiocollared males swimming across the Williston Reservoir, British Columbia during summer (56°N, 124°W—Lofroth 2001; approximately 6–8 km wide). Wolverines are highly vagile and roam over large home ranges averaging 303 km$^2$ for females and 797 km$^2$ for males, with some individuals occasionally dispersing over 300 km outside of their home range (Gardner et al. 1986; Inman et al. 2012). Likewise juvenile wolverines disperse an average of 51 – 60 km (males and females, respectively) from their natal range before establishing a new home range (Vangen et al. 2001). With both juvenile and mature wolverines routinely moving and dispersing around the landscape, there is a likely possibility that wolverines may attempt the passage to VI, although we are not aware of any documented observations. While the last confirmed sighting of a wolverine on VI was in 1992 (COSEWIC 2014), multiple unconfirmed sightings are reported every year, raising the possibility that the population still persists today. Wolverines are elusive and nocturnal, living in mostly remote regions, which adds to the possibility that some wolverines may still roam undetected on VI (Lofroth and Krebs 2007). Nevertheless, VI is a unique and unusual setting for wolverines and COSEWIC's geographic and historical criteria should still be considered when making recommendations on VI wolverines.

According to Moritz (1994), weak genetic divergence may be enough to classify populations as separate management units (MUs). However, Palsbøll et al. (2007) define MUs as populations that are demographically independent of one another. While this study lacks enough information

to determine if the modern VI population is demographically independent from the mainland, we might surmise that the (presumed) extremely low abundance is indicative of a sink population, and therefore not a separate MU. Generally, populations at the southern periphery of wolverine distribution may be more susceptible to extirpation, and therefore may need increased conservation efforts to minimize the continuing loss from their historical range (Kyle and Strobeck 2002). We recommend that the next step is to determine whether wolverines are still extant on VI and estimate their abundance and distribution accordingly. Further action could consider reintroducing wolverines from the mainland as VI does not appear to be a genetically unique population. However, intentional reintroduction would conflict with the ongoing conservation efforts toward the endemic and critically endangered Vancouver Island marmot (*M. vancouverensis*; Lloyd et al. 2019) and priorities should be weighed appropriately.

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## Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

**Supplementary Data SD1**.—Wolverine microsatellite loci data including allelic diversity ($N_A$), allelic richness ($A_R$), observed heterozygosity ($H_O$), and unbiased expected heterozygosity ($uH_E$) from Bridge River ($n$ = 15) and Vancouver Island ($n$ = 3), British Columbia.

**Supplementary Data SD2**.—Optimal $K$ selection and membership probabilities across $K$ = 2–4 for all STRUCTURE runs (Figs. S1–S4).

**Supplementary Data SD3**.—Performance assessments, scatter plots, and membership probabilities from the discriminant analysis of principal components across $K$ = 2–3 (Figs. S5 and S6), and assignment test using GenePlot (Fig. S7).

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