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Genetic subdivision and candidate genes under selection in North American gray wolves

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ABSTRACT

Previous genetic studies of the highly mobile gray wolf (*Canis lupus*) found population structure that coincides with habitat and phenotype differences. We hypothesized that these ecologically distinct populations (ecotypes) should exhibit signatures of selection in genes related to morphology, coat color, and metabolism. To test these predictions, we quantified population structure related to habitat using a genotyping array to assess variation in 42,036 SNPs in 111 North American gray wolves. Using these SNP data and individual-level measurements of 12 environmental variables, we identified six ecotypes: West Forest, Boreal Forest, Arctic, High Arctic, British Columbia, and Atlantic Forest. Next, we explored signals of selection across these wolf ecotypes through the use of three complementary methods to detect selection: F_{ST}/haplotype homozygosity bivariate percentile, `BayeScan`, and environmentally correlated directional selection with `Bayenv`. Across all methods, we found consistent signals of selection on genes related to morphology, coat coloration, metabolism, as predicted, as well as vision and hearing. In several high-ranking candidate genes, including *LEPR*, *TYR*, and *SLC14A2*, we found

variation in allele frequencies that follow environmental changes in temperature and precipitation, a result that is consistent with local adaptation rather than genetic drift. Our findings show that local adaptation can occur despite gene flow in a highly mobile species and can be detected through a moderately dense genomic scan. These patterns of local adaptation revealed by SNP genotyping likely reflect high fidelity to natal habitats of dispersing wolves, strong ecological divergence among habitats, and moderate levels of linkage in the wolf genome.

Introduction

By targeting genomic regions distinctly marked by positive selection, genes that are functionally important to individual fitness in natural populations can potentially be identified (Nielsen *et al.* 2007). Of particular interest are genomic regions having markers whose allele frequency variation is related to ecological differences among populations (Dobzhansky 1948; Hancock *et al.* 2008; Novembre & Rienzo 2009; Jones *et al.* 2012). However, allele frequencies are typically correlated between closely related populations due to shared population histories and gene flow, which potentially leads to elevated false positive rates (Coop *et al.* 2010). This problem can be circumvented in part by comparing multiple unlinked loci between populations since the effects of demography are genome-wide while selection is generally locus-specific (Nielsen 2005). Specific outlier loci can then be statistically identified and presumed to be in linkage disequilibrium (LD) with genes or other genomic features under selection (aka "tag" loci). Further, the broader characteristics of genes under selection can be determined through gene ontology (GO) enrichment methods, in which the frequency of certain categories of genes are measured relative to a background expectation (Primmer *et al.* 2013). Measurement of genome-wide patterns of variation using large scale SNP genotyping arrays is a crucial first step towards establishing evidence of local adaptation and illuminating the specific, functional

variants under selection in natural populations (e.g. Akey *et al.* 2002; Jones *et al.* 2012; Staubach *et al.* 2012; Pyhäjärvi *et al.* 2013). Consequently, we used a SNP genotyping array to explore evidence of local adaptation and identify candidate genes under selection in a highly mobile carnivore, the gray wolf (*Canis lupus*).

In North American gray wolves, there are genetically distinct populations which correspond to differences in ecological factors such as prey type and habitat; consequently, these populations have been considered "ecotypes" (Muñoz-Fuentes *et al.* 2009; Koblmüller *et al.* 2009; vonHoldt *et al.* 2011). Suggested reasons for this pattern included dispersal by individuals to habitats similar to their natal environment (natal homing) and the presence of discrete habitat and prey relationships (Geffen *et al.* 2004, Musiani *et al.* 2007). In coastal British Columbia, for example, wolves specialize on fish and small deer in near shore environments, tend to be smaller and more gracile than wolves elsewhere, and live in a wet temperate rainforest (Darimont *et al.* 2003). Previous studies have demonstrated that these wolves are genetically and ecologically distinct, even from inland British Columbia wolves (Muñoz-Fuentes *et al.* 2009). In addition, genetically distinct Arctic wolves are migratory, rather than territorial like most wolves, and follow barren-ground caribou during migratory movements of >1000 kilometers across cold, relatively dry, open terrain (Mech & Boitani 2003; Musiani *et al.* 2007). Similarly, genetically distinct wolves of the western forests of North America take larger prey, such as elk and moose, in heavily forested and mountainous terrain (Mech & Boitani 2003). These findings suggest the potential for divergent natural selection and resulting patterns of local adaptation (Hancock *et al.* 2008; Mullen & Hoekstra 2008; de Jong *et al.* 2012; Pujolar *et al.* 2014). Specifically, genes influencing morphologic features related to diet such as dentition, skull robustness and shape, vision (e.g. for open or closed terrain), locomotion (e.g. for migratory or territorial behavior),

metabolism and thermal regulation would be predicted to diverge among ecotypes. Variation in morphology has been found among North American wolves (Jolicoeur 1959; Musiani *et al.* 2007; Muñoz-Fuentes *et al.* 2009; O'Keefe *et al.* 2013) and diversification of cranial form corresponds to differences in prey size (Slater *et al.* 2009). Coat color and pattern likewise varies with paler pelage more common in Northern regions (Gipson *et al.* 2002; Musiani *et al.* 2007; Anderson *et al.* 2009). These phenotypic differences suggest functional categories of candidate genes that may underlie local adaptation in ecologically distinct populations of wolves.

In this study, we genotyped 111 wolves from across Canada and Alaska for variation in 42587 SNPs using Affymetrix v2 Canine SNP arrays. Our intent was to uncover population structure, and to identify genomic signals of selection and local adaptation in North American gray wolves. As the first step, we defined genetic units by quantifying population structure, isolation by distance and differentiation between subpopulations. To validate ecotype designations, we used a random forest model on high-resolution data collected on 12 environmental variables relating to temperature, precipitation, and vegetation. Next, we applied three approaches to identify SNPs showing signal of selection. First, we identified SNPs having outlier allele and haplotype frequencies between ecotypes using a composite statistic of F_{ST} and cross population extended haplotype homozygosity (XP-EHH) (Sabeti *et al.* 2007; vonHoldt *et al.* 2010). Second, we applied a model-based method (`BayeScan`) to identify SNPs that are significantly differentiated among populations, further suggesting diversifying selection (Foll & Gaggiotti 2008). Third, we applied a Bayesian approach (*`Bayenv`*; Coop *et al.* 2010) to identify significant correlations between SNPs and environmental variables. We took advantage of moderate levels of linkage disequilibrium in gray wolves (Gray *et al.* 2009) to identify candidate genes as those that are within 10 kilobases (kb) of an outlier SNP. Using GO enrichment analysis

and published functional data of specific candidate genes, we showed that selection may have acted on genes with morphological, phenotypic, and metabolic functions in relation to specific environmental variables. We also found significant genic SNPs and GO categories related to vision and hearing. Altogether, we demonstrated local adaptation in a highly mobile carnivore, and provided a set of >500 candidate genes for verification in a comprehensive resequencing study using a gene capture array (Schweizer *et al.*, submitted).

Methods

Sample selection and genotyping

The samples that we genotyped were selected from a set of gray wolves used in previous studies (Carmichael *et al.* 2007; Musiani *et al.* 2007) with additional tissue samples obtained from the University of Alaska Museum (Fairbanks, AK) and from P. Paquet (University of Victoria, Canada) to maximize geographic representation in northern Canada and Alaska. All samples were collected under permits granted to researchers at these institutions (Carmichael *et al.* 2007; Musiani *et al.* 2007; Muñoz-Fuentes *et al.* 2009). Forty-five samples were previously genotyped on the genome-wide Affymetrix v2 Canine SNP array (vonHoldt *et al.* 2010). We genotyped an additional 87 samples on the same SNP arrays following the manufacturer's protocol (Supporting Information).

After array hybridization and scanning, genotypes were called using the MAGIC algorithm (Boyko *et al.* 2010) in reference to the dog genome (CanFam2; Lindblad-Toh *et al.* 2005). The majority (>98%) of the SNPs on the array were ascertained within the boxer and a small number of dog breeds (Lindblad *et al*. 2005; vonHoldt *et al*. 2010), and consequently, were

monomorphic in wolves. As in previous studies (vonHoldt *et al*. 2010, vonHoldt *et al*. 2011, Pilot *et al*. 2014), after filtering (Supporting Information), a total of 42587 SNPs were retained for analysis (henceforth referred to as 42K SNPs). Of these SNPs, 26108 (61%) were within 10 kb upstream or downstream of a gene. Although this represents a biased sample of the genome overall, it is appropriate for identifying outliers with regard to genic regions (e.g. vonHoldt *et al*. 2010; Pilot *et al.* 2014). Fourteen closely related individuals were identified and removed from further analyses, following methods described previously (vonHoldt *et al.* 2011), and we used the remaining 111 individuals for analysis. Because of the potential for LD biasing our results, we also generated a reduced data set of 22084 SNPs that were not in high LD due to physical proximity (henceforth referred to as 22K LD-pruned; Supporting Information). This 22K LD-pruned dataset was used for population genetic analyses where indicated below as this provides unlinked markers representing independent assessments of genome history (e.g. vonHoldt *et al*. 2010, 2011; Pilot *et al*. 2013; Sazzini *et al*. 2014; Zhang *et al*. 2014). The 42K set was used for selection tests as it provides greater density of SNPs in genic regions that may be under selection. It is possible that the use of a dog SNP array in wolves may impose ascertainment bias, especially in studies comparing dogs to wolves and other canine species (vonHoldt *et al.* 2010; vonHoldt *et al.* 2011). However, this bias is expected to be consistent within wolves, and the large number of varying SNPs within our samples supports the use of this array for an intraspecific study.

Population structure

In order to determine the population structure within our samples, we first used `STRUCTURE` (Pritchard *et al.* 2000; Falush *et al.* 2003) to identify genetic clusters of individuals. Using the 111-individual 22K LD-pruned data set, we ran `STRUCTURE` v2.3.4 with 20000 burn-

in iterations followed by 50000 sampling iterations for K = 1 through 10, assuming correlated allele frequencies under the admixture ancestry model. Each run was performed 10 times, and the ΔK statistic of Evanno *et al.* (2005) was calculated to help determine the most appropriate number of genetic clusters using `Structure Harvester` v0.6.93 (Earl & vonHoldt 2012). We used the *greedy* algorithm within `CLUMPP` v1.1.2 (Jakobsson & Rosenberg 2007) to account for variation in cluster labels across the 10 random iterations of `STRUCTURE`. Individuals that had > 50% assignment to a single genetic cluster were considered part of a population, and individuals with a lower percentage assignment were characterized as "admixed".

We performed a principal components analysis (PCA) (Patterson *et al.* 2006) using `SMARTPCA` within `EIGENSTRAT` v3.0 (Price *et al.* 2006) for the 111-individual 22K LD-pruned SNP data set. To measure the degree of genetic differentiation between clusters identified by non-admixed individuals (n=94), we used custom scripts to calculate Weir and Cockerham's (1984) θ, an estimator of Wright's (1951) F_{ST} (and henceforth referred to as F_{ST}), across all clusters and between each pair of clusters. Finally, to further visualize patterns of population structure, majority-rule neighbor-joining trees based on allele-sharing distances, which were calculated using `PLINK` (Purcell *et al.* 2007), were constructed using the package `ape` 3.1-2 in `R` (Paradis *et al.* 2004; http://www.R-project.org). Trees were generated using the 22K LD-pruned SNP set, with 1000 bootstraps, then visualized within the `ape` package.

Isolation by distance and spatial autocorrelation

To assess isolation by distance (IBD), Mantel tests were performed to compare pairwise geographic distances with genetic distance, D_{IBS}, calculated within `PLINK` for the 42K SNPs. The Mantel analysis was performed with the `vegan` v.2.0-10 package (Oksanen *et al*. 2013) in `R`

using 1000 permutations to test the correlation between genetic and Log_{10}–transformed geographic distance. Using the same data within GENALEX v6.501 (Peakall & Smouse 2006), we measured spatial autocorrelation within 61 even distance classes of 100 km each. Significance was assessed using 9999 permutations, and the 95% confidence interval around the correlation *r* was determined using 9999 bootstraps.

Environmental layers and habitat classification

We acquired environmental characteristics for each individual using georeferenced environmental layer datasets (Hijmans *et al.* 2005) consisting of variation in annual means, extremes, and seasonal variation in temperature and precipitation, measured at 1-km spatial resolution. We used a set of 12 environmental variables: eight WorldClim variables were previously determined to maximize the variation within North America while minimizing correlation (Harrigan *et al.* 2014), and four satellite/radar variables were added after minimizing the Pearson correlation among a larger set, as in Harrigan *et al.* (2014). The 12 variables measure temperature (annual mean temperature, mean diurnal temperature range, temperature seasonality, maximum temperature of warmest month, minimum temperature of coldest month), precipitation (annual precipitation, precipitation seasonality, precipitation of coldest quarter), vegetation (percent tree cover, normalized difference vegetation index, and land cover category), and altitude. This set of environmental variables includes those such as precipitation that have been demonstrated to significantly affect wolf population structure (Geffen *et al.* 2004) and morphology (O'Keefe *et al.* 2013).

To test whether our population groupings, as determined through genetic tests alone, were ecologically different and could be justified as unique ecotypes for downstream methods,

we used a tree classification method called random forest (Breiman 2001) by utilizing the `randomForest` package (Liaw & Wiener 2002) in `R`. This test uses environmental data for each individual, in conjunction with our suggested population assignment based on genetic data, to test how well each individuals can be assigned to a group based on environmental data alone. The software uses a subset of individuals to train the model, and then attempts to assign "test" individuals to a group. Accuracy is measured by how often the model correctly assigns test individuals to the group specified for them. Assignment of individuals to populations using the 12 environmental variables had an accuracy of 82.98% from 50000 trees. Accuracy was highest for British Columbia and Atlantic Forest populations, and most errors occurred when assigning individuals to the West Forest or Boreal Forest populations and to the Arctic or High Arctic populations. This difficulty in assigning individuals from these populations was also observed in `STRUCTURE` assignment tests (see *Results*). Based on the close correspondence of populations with unique environments, we subsequently classified them as "ecotypes".

Detection of ecotype-specific selective sweeps

In order to detect markers under selection within each ecotype, we grouped wolves based on `STRUCTURE` and random forest results. Only non-admixed wolves were analyzed (n = 94) so as to focus on detecting molecular evidence for local adaptation to specific habitats. We employed the joint F_{ST} and cross-population extended haplotype homozygosity test (`XP-EHH`; (Sabeti *et al.* 2007; vonHoldt *et al.* 2010), which has been used previously to identify selective sweep regions in multiple species (e.g. Sabeti *et al.* 2007; vonHoldt *et al.* 2010). The XP-EHH test uses the difference in haplotype length between two populations to identify regions that have undergone a hard selective sweep in one population if they show an extended haplotype in that population but not the other. The XP-EHH test requires a reference or ancestral population for

the population being assessed. However, there is no straightforward ancestral population for each of the ecotypes identified here. Therefore, we compared each ecotype to its most closely related population, as determined by pairwise F_{ST}, and additionally to a pseudo-population consisting of all other ecotypes combined. Consequently, we identified regions that diverged in each ecotype since splitting from the most recent ancestor, or regions that were specific to that ecotype in comparison to all other populations. Similar approaches have been applied in more recent resequencing studies (Yi *et al.* 2010; Carneiro *et al.* 2014).

XP-EHH was calculated between each comparison pair mentioned above. This analysis requires data with known haplotype phase, so data were phased using `fastPHASE` software (Scheet & Stephens 2006) with subpopulations labeled according to their genetic population group (Supporting Information). Using developed methods (vonHoldt *et al.* 2010), we computed the empirical percentile for normalized F_{ST} and XP-EHH values associated with each SNP. A bivariate percentile score was calculated from the product of the F_{ST} and XP-EHH percentiles to obtain a single summary of the strength of the two signatures. If two or more SNPs were in the 95th percentile of the bivariate percentile score and were spaced <300 kb apart, they were joined into a single cluster (vonHoldt *et al.* 2010). We ranked clusters by the number of SNPs they contained then by the bivariate percentile score of the central SNP. We selected the top 5% of empirical outlier clusters from each pairwise population, and then took the union of the two approaches (comparison to the population with the smallest F_{ST} and comparison to all other populations). We also examined candidate sweep regions with a bivariate percentile score above 99.5th percentile.

Model-based directional and balancing selection

To assess directional selection, we used the Bayesian method implemented in `BayeScan v2.1` (Foll & Gaggiotti 2008). `BayeScan` tests whether subpopulation-specific allele frequencies, measured by an F_{ST} coefficient, are significantly different from the allele frequency within the common gene pool, and assigns a posterior probability (alpha) to a model in which selection explains a difference in allele frequencies better than a null model. A positive alpha indicates population-specific directional selection while a negative alpha suggests balancing or purifying selection. Given that `BayeScan` may suffer from elevated false positive rates under IBD and range expansion (Lotterhos & Whitlock 2014), and that balancing or purifying selection is especially prone to such issues (Lotterhos & Whitlock 2014), we focused on directional selection. Additionally, `BayeScan` was run using prior odds of 10, 1000, or 10000 (Lotterhos & Whitlock 2014). Higher prior odds may reduce the false positive rate at the expense of identifying true loci under selection (Foll & Gaggiotti 2008). A false discovery rate (FDR) of 0.05 was used, with the caveat that although this reduces the number of false positives, true signals of selection may be missed (Foll & Gaggiotti 2008).

Environmentally correlated selection

We used a Bayesian method (`Bayenv`) to identify allele frequencies that correlate with environmental variables (Coop *et al*. 2010). In this approach, the empirical covariance in allele frequencies between geographically varying populations is initially estimated from a set of random markers (Hancock *et al.* 2008; 2010; Coop *et al.* 2010; Gunther & Coop 2013). Next, a Bayes Factor (BF) is assigned to each SNP of interest as a measure of how well the allele

frequency of that SNP co-varies linearly with an environmental variable above the null model based on population structure alone.

In order to build a covariance matrix for the joint distribution of allele frequencies across populations, 10000 SNPs were randomly chosen out of the full 42K SNP set after excluding SNPs that were out of Hardy-Weinberg equilibrium ($p<0.01$, exact test) using `PLINK`. These filters were applied to SNPs for the background covariance matrix as recommended by the authors (Coop *et al.* 2010), but all 42K SNPs were tested in the selection mode. Covariance matrices output by Bayenv after every 20000^{th} iteration were averaged over a total of 500000 iterations. Following author recommendations (Coop *et al.* 2010), we compared the average correlation matrix (generated from the average covariance matrix with the *cov2cor* function in R) to our pairwise F_{ST} matrix for unusually high or low correlations, which might mean the MCMC model had not stabilized.

The selection mode of `Bayenv` was run separately for each SNP in the full 42K set with a total of 12 environmental variables and 100000 iterations for each SNP. Each variable was normalized following author recommendations (Coop *et al.* 2010). `Bayenv` was run 10 times, since many MCMC sampling methods are sensitive to the initial conditions (Coop *et al.* 2010; Blair *et al.* 2014), and the final matrix of BFs was averaged over these 10 independent runs. For each of the 12 environmental variables, the empirical percentile of the $\log_{10}$ BF of each SNP was calculated. Both the top 5% and top 0.5% of outlier SNPs were candidates for further analysis (see below). For outlier SNPs, we plotted the mean value of the environmental variable within each population against the allele frequency for each population. `Bayenv` uses allelic correlations between populations as a proxy for geographic distance in assessing the significance of environmental association with individual SNPs. To further test the influence of geographic

distance on SNP associations, we used distance-based redundancy analysis (dbRDA; Legendre and Anderson 1999). Specifically, for SNPs with BF>3, we used dbRDA to assess whether the effect of environmental variation on genetic distance was significant conditional on geographic distance. This approach has been widely used for similar questions in many species, including wolves (e.g. Geffen *et al*. 2004; Carmichael, *et al*. 2007; Musiani *et al*. 2007; Lasky *et al*. 2012; Lasky *et al*. 2015). We performed dbRDA and assessed significance with the `capscale` function within the `vegan` package in `R`, conditioning upon the latitude and longitude.

Candidate gene identification and gene ontology enrichment analysis

Using curated gene annotations from UCSC and Ensembl and accounting for different dog assembly versions (Freedman *et al.* 2014), we determined if there was any gene within 10 kb of each candidate SNP or sweep region. SNPs at this distance would likely be in LD with that gene (Gray *et al.* 2009). Gene lists from each of the three selection tests were tested for significant enrichment of GO categories using `gProfiler` (Reimand *et al.* 2007; 2011). After correction for multiple testing using the Benjamini-Hochberg FDR, we examined significant categories ($P \leq 0.05$) with a minimum of two genes (Zhang *et al.* 2014). We also tested for an excess of genic SNPs among outliers using a one-sided conditional exact test (Agresti 2002) in `R`.

Phenotypic-genotypic association

For 33 of our wolf samples, we also had information on coat color phenotypes. Twenty-three of these wolves were sampled in a previous study on coloration and ecotype variation (Musiani *et al.* 2007) and were subsequently genotyped and included here. An additional 10 previously genotyped wolves from Yellowstone National Park (vonHoldt *et al.* 2011) that were

not included in the analyses above because they represent translocated individuals, were included in the coat color analysis yielding 11 white, 11 black, and 11 gray (wild type) individuals. In order to test for associations between SNPs near coat color genes and phenotypic variation within our samples, we performed a case/control association test using both the Fisher's exact test for allelic association (--fisher) and the full model testing for differences in any genotypes, with permutations for assigning significance (--model --cell 0 –perm) within PLINK. We implemented this for both white versus non-white coat color and black versus non-black coat color.

Results

Population structure and ecotypes

We observed notable population structure among our samples (Figure 1A-D). STRUCTURE runs showed the highest peak in ΔK values at K=3 and K=7 (Figure S1). At K=3, there were distinct forest, arctic, and Atlantic groups (Figure 1C). Given the expansive geographic and environmental range represented by our samples (Figure 1A), we chose to examine higher values of K. Increasing values of up to K=6 appeared to separate different wolf ecotypes and confirm previous STRUCTURE groupings (Carmichael *et al.* 2007; vonHoldt *et al.* 2011). K=7 was not more informative with regard to geographic or habitat groupings and increasing K past 7 yielded no additional clusters to which more than three individuals were strongly assigned (i.e. > 50%). We therefore used K=6 genetic clusters for subsequent analysis. The six clusters were geographically coherent (Figure 1A), had high average assignment within each genetic cluster (84.5% ± 6.9%), and corresponded to specific habitats as found previously using microsatellite and SNP data: West Forest, Boreal Forest, Arctic, High Arctic, British

Columbia, and Atlantic Forest (Carmichael *et al.* 2007; Muñoz-Fuentes *et al.* 2009; vonHoldt *et al.* 2011). After removal of two individuals whose STRUCTURE assignments showed they were migrants, we found that all six subpopulations, or ecotypes, were well circumscribed (Figure 1A).

Genetic differentiation of the 22K LD-pruned SNPs measured between all ecotypes was moderate, with global $F_{ST} = 0.09$. Pair-wise F_{ST} ranged from 0.0154 between Boreal Forest and West Forest ecotypes to 0.1128 between High Arctic and British Columbia ecotypes, with mean pair-wise $F_{ST} = 0.07$ (Figure 1B). The British Columbia ecotype appeared most distinct by this measure, showing high pairwise F_{ST} estimates with other ecotypes (Figure 1B).

There was high congruence between the STRUCTURE subpopulation assignments and their pattern of clustering by PCA. The same geographically coherent groups appeared clustered according to their scores on the first two axes, PC 1 and PC 2 (Figure 1D; Figure S2). The first and second axes accounted for 4.2% and 3.8%, respectively, of the observed genetic variation. Within this PC space, admixed individuals were intermediate between the ecotypes for which their assignment was split in STRUCTURE analysis (Figure 1C-D). Results from neighbor-joining analyses generally supported structure and admixture population assignments with 100% support for all nodes in trees generated using 22K LD-pruned SNPs (Figure S3).

As described in the methods, we used a random forest approach to confirm our ecotypes and to identify the environmental variables most significant in distinguishing them (Figure 2). The ecotypes demonstrated extensive variation in annual precipitation, mean diurnal temperature range, elevation, and maximum temperature (Figure 2A), and the relative importance of each of the 12 environmental variables to distinguishing ecotypes can be visualized (Figure 2B). The

Pearson correlations for each environmental variable with latitude and longitude are provided (Table S1).

Isolation by distance and spatial autocorrelation

We found a significant correlation between geographic distance and genetic distance, D_{IBS}, across the 111 individual 42K SNP data set ($r = 0.560$; Mantel test $P = 0.001$; Figure 3). This correlation was slightly weaker among wolves located more than 300 km apart ($r = 0.537$; Mantel test $P = 0.001$; Figure 3) and among those separated by shorter geographic distances ($r = 0.330$; Mantel test $P = 0.002$) (Figure 3). Spatial autocorrelation analysis showed a significant positive spatial autocorrelation in distance classes from 0 km to 1350 km (Figure S5). Between 1550 km to 4850 km, there was a significant slightly negative autocorrelation, and beyond 4850 km the trend showed negative spatial autocorrelation, but without significance (Figure S5). Given that spatial autocorrelation and IBD were not substantial, and are partially accounted for in the `Bayenv` approach (Coop *et al*. 2010), we chose not to discuss the results further below.

Selective sweeps within ecotypes (F_{ST}/*XP-EHH*)

The numbers of candidate genes from the joint F_{ST}/XP-EHH selection scan outlier regions are provided in Table S2 and the coordinates of the top 60 clusters for each ecotype comparison are provided, along with their size, F_{ST}/XP-EHH percentile and genes in Table S3. Only British Columbia wolves showed a significant increase in the proportion of genic SNPs in the top 5% of outlier regions compared to the full data set (one-sided exact conditional test, 1 degree freedom, $P < 0.05$). GO enrichment tests performed on each of these sets of genes in `gProfiler` identified several enriched categories (Table S2, Table S4, Table S5). GO categories relating to skeletal morphology, vision, organismal system, metabolism, immunity,

response to environment, and dentition were enriched in all ecotypes, although the specific GO categories for each ecotype were usually different, implying that slightly different sets of genes were enriched (e.g. Table 1, Table S4, Table S5).

Several high-ranking joint F_{ST}/XP-EHH selective sweep regions contained notable candidate genes (Table S3, Supplemental Information). Although an intention of this study was to generate candidates for resequencing (Schweizer *et al.* in review), we understand that many may be false positives that will fail verification upon further study. We discuss some of our most promising candidates below, and provide detailed gene descriptions for other top candidates in the Supplemental Information. A top candidate gene for morphology within the West Forest ecotype was *NOTCH2* (*Notch (Drosophila) Homolog 2*), which included GO categories such as "positive regulator of the bone morphogenetic protein (BMP) signaling pathway" and "limb morphogenesis". Within the West Forest wolves, the cluster containing *NOTCH2* contained three SNPs above the 95th percentile, including one SNP with a joint percentile of 99.9%. A top candidate gene within the Boreal Forest ecotype was *GDF5* (*Growth Differentiation Factor 5*), which encodes a protein that is a member of the BMP family (Figure 4A) (Nie *et al*. 2006). Two SNPs within the cluster containing *GDF5* together ranked at the 99.5th percentile. A high-ranking candidate region within High Arctic wolves contained a single gene, *KIT* (v-*kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog*), which is an essential cell-surface receptor in the melanogenesis pathway (Wehrle-Haller 2003). This sweep region contained a single SNP with a joint percentile of 96.1% (Figure S6). A high-ranking sweep cluster within British Columbia wolves contained *WNT5A* (*Wingless-Type MMTV Integration Site Family, Member 5A*), a gene which plays a critical role in determining size during murine tooth development (Figure S7; Cai *et al.* 2011). Within the Atlantic Forest wolves the highest ranking cluster (4

SNPs, max. percentile: 97.9%) contained *PLEKHB1* (*Pleckstrin Homology Domain Containing, Family B Member 1*), which is involved in retinal development in mice (Wan *et al.* 2011), and *MRPL48* (*Mitochondrial Ribosome Protein L48*), which showed evidence in Antarctic icefish of gene duplications to increase mitochondrial function (Coppe *et al.* 2013).

Population-specific directional selection

The `BayeScan` algorithm identified 77 SNPs with a value of alpha above the FDR cut-off of 0.05 using the default prior odds of 10 (Figure 5). Forty-four of these SNPs were within 10kb of an annotated gene (Table S6). Of the 27 annotated genes near SNPs with a positive alpha (indicating diversifying selection), GO analysis identified a single significantly enriched category of "auditory receptor cell differentiation", and KEGG pathways related to oxytocin signaling and cardiac muscle contraction (Table 2; Table S7). When we used prior odds of 1000, a single SNP near *ANXA10* (discussed below) was significant, and when we used prior odds of 10000 there were no significant SNPs.

The top candidate gene for positive selection from `BayeScan` was *ANXA10* (*Annexin A10*), a protein coding gene for which the function is not yet known (Table S6). The only significantly enriched GO category contained two interesting candidate genes. The first gene, *PCDH15* (*Protocadherin-related 15*) plays a crucial role in upkeep of normal cochlear and retinal function (Le Guédard *et al.* 2007). The second candidate gene within that GO category was *CUX1* (*Cut-like homeobox 1*), which plays a broad role in mammalian development by regulating morphogenesis (Lizarraga *et al.* 2002; Sansregret & Nepveu 2008).

Correlation between SNPs and environmental variables

Our samples of North American wolves were distributed across a variable environment (Figure 2). Using `Bayenv` we found multiple significant outlier SNPs for each of the 12 environmental variables (Figure 6; Figure S9). Across all 12 sets of outlier loci, a single vegetation variable (normalized difference vegetation index) showed a significant enrichment of genic SNPs in the top 5% (Fisher's exact test; $P = 0.0326$) (Table S8). Nonetheless, there were several significantly enriched GO categories relating to hearing, morphology, pigmentation, smell, and organismal system for each of the 12 environmental variables we examined (Table 3, Table S9, Table S10). For example, morphological categories such as "anatomical structure development" and "anatomical structure morphogenesis" were enriched in 11 and 10, respectively, of the temperature, precipitation, vegetation, and elevation variables (Table 3). Organismal system categories involved in "calcium ion binding" and "locomotion" were enriched in the majority of environmental variables, whereas "blood circulation" was enriched with mean annual temperature and all of the vegetation and elevation variables. Two categories related to hearing were enriched as well (Table 3). Of particular interest were GO categories related to pigmentation that were significantly enriched with annual mean temperature and vegetation variables. Results from dbRDA using SNPs with a BF>3 (n=1658) showed that 10 out of 12 environmental variables had a significant effect ($P \leq 0.10$) on genetic distance, even after controlling for geographic distance as measured by latitude and longitude (Table S11).

Here, we conservatively present SNPs with high support as measured by a BF>3 and that tag genes which can be interpreted with regard to specific ecological characteristics of populations. Several top-ranking SNPs from `Bayenv` were located near genes implicated in energy regulation, metabolism, and water balance, and show high correlation with environmental

variables. *LEPR* (*Leptin Receptor*) is a receptor for the adipocyte-specific hormone leptin, and is involved in obesity (Chua *et al.* 1996) and cold tolerance (Hancock *et al.* 2008). A SNP located less than 1kb upstream of the start codon of *LEPR* ranked above the 99.9th percentile for land cover classification (BF=106.8) (Figure S10). Located less than 1kb downstream of *LIPG* (*Endothelial Lipase*) was a SNP above the 99.9th percentile (BF=71.1) for temperature seasonality. *LIPG* regulates lipid levels, specifically levels of HDL (Edmondson *et al*. 2009). An intronic SNP in *SLC14A2* (*Solute Carrier Family 14, Urea Transporter, Member 2*) ranked above the 99.5th percentile in elevation (BF=144.1). *SLC14A2* plays a major role in water and salt balance through the urinary concentration mechanism (reviewed in Smith & Fenton 2007).

A number of top-ranking SNPs from `Bayenv` were also located near candidate genes implicated in the BMP pathway regulation of skeletal and eye development. Several genes in this pathway, including *BMP1*, *BMP4*, *BMP6*, *BMP7*, *BMP10*, *BMPER*, and *GDF5* (Bragdon *et al.* 2011), were in the top 95th percentile for environmental variables related to temperature, precipitation, and elevation (e.g. Figure 4B). Additional SNPs above the top 99th percentile tagged two *FGF* (*Fibroblast growth factor*) genes, which are implicated in craniofacial skeletal formation in humans, dogs, and mice (e.g. Hünemeier *et al.* 2013). The first gene, *FGF3*, was tagged by a downstream SNP that was an outlier for percentage tree cover (BF=3.17), and the second gene, *FGF14*, was tagged by an intronic SNP highly ranked for mean diurnal temperature range (BF=5.46).

Finally, SNPs near genes within the pigmentation pathway were outliers. *TYR* (*Tyrosinase*) encodes an enzyme crucial to the conversion of tyrosine to melanin (Beermann *et al.* 2004). A SNP located in the intron of *TYR* ranked above the 99th percentile for precipitation seasonality (BF=4.07) (Figure S10). *OCA2* was tagged by a SNP 7kb downstream that was an

outlier for mean diurnal temperature range (BF=5.3, 99.7th). Finally, the receptor *KIT* was near a high-ranking SNP above the 95th percentile; *KIT* was tagged by a SNP (BF=3.05, 99.2th) for precipitation seasonality.

Overlap of candidate genes

GO enrichment of all genes within the top 0.5% of outliers from either only `Bayenv` and F_{ST}/XP-EHH, or `Bayenv`, F_{ST}/XP-EHH, and `BayeScan` (FDR≤0.05), identified significant enrichment in GO categories of "anatomical structure development", "locomotion", "sensory perception", "regulation of cation channel activity", and several human phenotype categories related to abnormal morphological development, increased body weight, and hair color (Table S12, Table S13). At the 5% level of candidate gene significance, there were 276 significantly enriched GO categories, of which at least 21 related to morphology (e.g. "limb development"), four related to movement (e.g. "locomotion"), nine related to sensory perception and stimulus (including "eye development"), 34 related to channel or transporter activity (e.g. "ion channel activity"), and nine were related to muscle (e.g. "muscle tissue development"; Table S14). Consequently, 77 of 276 GO categories (28%) could be interpreted as consistent with our hypotheses for local adaptation

At the 0.5% level, there was overlap between each pair of tests (except for between `BayeScan` and F_{ST}/XP-EHH), but no overlap among all three tests (Figure S11). However, at the 5% level, there was overlap between each pair of tests, and 14 genes overlapped among all three tests (*ANK2*, *AZIN1*, *BCAS1*, *BTN1A1*, *CACNA2D3*, *CCDC33*, *FOXK1*, *KSR2*, *LOC100685844*, *LOC100855656*, *LOC100855681*, *LOC100856364*, *LRRC16A*, *MPPED2*).

Out of interest, we also determined the level of overlap between our candidate genes and those from independent studies either focusing on wolves (Pilot *et al.* 2014; Zhang *et al.* 2014) or focusing on environment-related selection in humans in North America (Hancock *et al.* 2008) (Figure 7). These candidate genes were identified using the Affymetrix canine SNP array and `BayeScan` (Pilot *et al.* 2013), a scan measuring F_{ST} and nucleotide diversity in whole genome sequences (Zhang *et al*. 2014), or a seed gene and network approach with tag SNPs and `Bayenv` (Hancock *et al*. 2008). The numbers of candidate genes pooled across our three methods at the top 5% and 0.5% level were 6225 and 1035, respectively. We reasoned that overlap of our genes with candidates from other studies might strengthen our case for selection acting on these genes. At the 5% level, the gene *CACNA2D3* (*calcium channel, voltage-dependent, alpha 2/delta subunit 3*) was common to the three selection tests applied here and both Pilot, *et al.* (2014) and Zhang, *et al.* (2014). *CACNA2D3* is involved in voltage-gated calcium channel activity and six different SNPs near *CACNA2D3* ranked above the 95th percentile in temperature, precipitation, and vegetation. The gene *AZIN1* (*antizyme inhibitor 1*) also overlapped between this study and that of Zhang, *et al.* (2014). Antizymes catalyze a rate-limiting step in polyamine biosynthesis and are crucial to cell development (Coffino 2001). Eight genes (*CLOCK, PON1*, *LEPR*, *PPARGC1A*, *EPHX2*, *TCF7L2*, *PTK2B*, *SCARB2*) overlapped between our top 5% of `Bayenv` results and those of Hancock, *et al.* (2008).

Phenotypic-genotypic association

For white coat color, we identified an intronic SNP of *MITF* (*P*: 9.8×10^{-3}), a modulator of melanocyte-related genes such as *KIT* and *KITLG* (Goding 2000) and the gene implicated in white spotting in many dog breeds (Karlsson *et al.* 2007; Schmutz *et al.* 2009; Vaysse *et al.* 2011). For black coat color, the most significant SNP tagging a pigmentation gene was within

the intron of *TYR* (*P*: 2.8×10^{-2}). These genes were tagged by SNPs above the 95th percentile for at least one environmental variable in `Bayenv`.

Discussion

Population structure and genetic differentiation across populations

Our analysis of population structure of North American gray wolves revealed six major clusters that were associated with unique habitats (Figures 1, 2). These results were concordant with previous large-scale studies in wolves using microsatellites or SNPs (Geffen *et al.* 2004; Carmichael *et al.* 2007; vonHoldt *et al.* 2011). We found that mainland tundra wolves were highly admixed and contained genetic components of both Boreal Forest and Arctic subpopulations (Figure 1A, 1C). Additionally, the PCA did not provide any evidence of a mainland tundra subpopulation, as found by Carmichael *et al.* (2007) (Figure 1D). This discrepancy in population structure may reflect differences in geographic sampling and the several orders of magnitude greater number of markers assayed in our study. We confirmed previous studies finding that British Columbia wolves are genetically and ecologically distinct (Muñoz-Fuentes *et al.* 2009). Our results highlight the differentiation of the British Columbia ecotype, which was one of the first to appear in `STRUCTURE` analysis as a separate group at increasing K values (K=4), and also the population separated on PC1 (Figure S2). Additionally, we found that Atlantic Forest wolves are genetically and ecologically distinct. In PCA and structure analyses this population was more distinct than the British Columbia wolves, and random forest classification had 87.5% accuracy. Using data from 12 environmental variables shown to be important in discriminating North American habitats (Harrigan *et al.* 2014), we distinguished six environmentally distinct populations using a random forest classification

method (Figure 2). Precipitation was the climate variable that most strongly associated with the differences among ecotypes, which agrees with a previous analysis based on microsatellite loci and mtDNA (Geffen *et al.* 2004). Mean diurnal temperature range and maximum temperature of warmest month were also significant, which was a novel finding here. Random forest models had lower accuracy when assigning individuals to either West forest or Boreal forest, and to High Arctic or Arctic, which paralleled the moderate level of admixture identified from genetic data alone (Figure 1). Further exploration of environmental differences among wolf ecotypes, especially those that are related to threshold responses in organisms (e.g. Fitzpatrick & Keller 2014), is of interest but beyond the scope of the current analysis. In summary, through the use of population structure and environmental classification methods, we demonstrated that environmental influences dominate population structure in wolves, with weaker trends evidenced by slightly positive autocorrelation to 1350 km. Such weak patterns of differentiation with distance might be expected for a highly mobile species (Geffen *et al.* 2004).

Candidate genes for morphology

Given the potential problem of over analyzing candidate genes and "storytelling" (Pavlidis *et al.* 2014), we keep discussions brief and await further confirmation by resequencing in a second manuscript (Schweizer *et al*. in review). We focus on candidate genes that have high support as outliers from multiple independent selection tests (each with their own unique assumption) or are especially convincing candidates given wolf natural history. Both GO (Table 1; Table 3) and candidate gene analyses (Figure 4; Figure S10) suggested that selection on morphological pathways has occurred in North American wolves, as we predicted. Several genes within the BMP pathway are top candidates in F_{ST}/XP-EHH and `Bayenv`. We found that *GDF5, BMP7*, and *NOTCH2* were located in candidate selective sweep regions in Boreal Forest wolves,

British Columbia wolves, and West Forest wolves, respectively. Mutations within *GDF5* are associated with skeletal developmental disorders (Bragdon *et al.* 2011), functioning *BMP7* is necessary for normal cartilage and eye development (Bragdon *et al.* 2011), and mouse knockout experiments have shown that *NOTCH2* is critical for proper chondrocyte and bone development (Kohn *et al.* 2012). Within additional pathways for skeletal mineralization or limb development, we found top clusters for *ALPL* in Arctic wolves, *WNT5A* in British Columbia, and *WNT5B* in Atlantic Forest using F_{ST}/XP-EHH (Figure S7). Mouse knockout experiments have shown that *WNT5A* and *WNT5B* are critical for chondrocyte proliferation and tooth development (Lin *et al.* 2011; Cai *et al.* 2011). SNPs either within the introns of these genes or in close proximity were also significant outliers in our environmental analyses of selection with `Bayenv`. For example, one of the top candidate sweep regions within the Boreal Forest wolves contained multiple SNPs at the 99.5th percentile near *GDF5* (Figure 4A) and was highly correlated with annual mean temperature (Figure 4B). If climate is influencing prey type and availability, then wolves in differing environments may have evolved consequentially divergent skull morphologies. Genes that are critical for tooth development, for example, may be under selection in response to diets consisting of smaller prey such as deer or fish, rather than elk or moose, which may require special dental adaptations or cranial bite force (Slater *et al.* 2009). In general, we found that SNPs located near or within genes that are fundamental to bone, skeletal, and muscle development were highly correlated with both precipitation and temperature variables. It follows that a recent study that revisited skull measurement data collected on almost 300 wolves from all over North America (O'Keefe *et al.* 2013) found distinct trends in morphological variation, with higher mean body size at higher latitudes, and identified precipitation as a key factor driving the variation in cranial morphology.

Candidate genes for coloration

In the GO analyses of `Bayenv` results, we observed significant enrichment of categories related to pigmentation, melanin biosynthetic process, and melanosome membrane for environmental measures of temperature and vegetation (Table 3, Table S9, Table S10). The lack of similar GO categories in the F_{ST}/XP-EHH analysis may indicate that within a single ecotype multiple candidate pigment genes may not be under selection such that a GO analysis would be of limited use. Alternatively, if pigmentation is a result of polygenic selection and genes have not undergone a classic selective sweep, then XP-EHH would be unlikely to detect selection. Using F_{ST}/XP-EHH we did identify a candidate sweep region within High Arctic wolves that contained a single SNP tagging a single pigmentation gene, *KIT* (Figure S6). *KIT* is a key component in the melanogenesis pathway, and given the low frequency of black wolves in the High Arctic (Musiani *et al.* 2007), may be involved in the higher frequency of light coat color. For *OCA*2, *TYR*, *ASIP*, and other genes within the pigmentation pathway, we observed allele frequency changes of SNPs with BF>3 across environmental variables (Figure S10). Several of these genes have been associated with color polymorphisms within wild vertebrate populations (reviewed in Hubbard *et al.* 2010). Within the pathway by which melanin pigment is produced, tyrosinase is the rate-limiting enzyme, and several mutations within *TYR*, the gene encoding tyrosinase, have been identified causing coat colors in mice along the spectrum of fully pigmented to albino (reviewed in Beermann *et al.* 2004). We suspect that similar mechanisms may occur in wolves, especially since we found significant association of a SNP in *MITF* and a second SNP in *TYR* with white and black coat color, respectively. These pigmentation candidate genes warrant further study, perhaps through resequencing to identify functional variants, or measuring gene

expression differences in wolves of known phenotype (e.g. Hoekstra *et al.* 2006; Linnen *et al.* 2013).

Candidate genes for metabolism, vision, and hearing

We identified high-ranking SNPs tagging genes that may affect metabolic performance, as well. A SNP located less than 1kb upstream of *LEPR* was above the 95th percentile in vegetation, temperature, and precipitation variables. *LEPR* has been implicated in cold tolerance and cold adaptation, and here, the SNP tagging *LEPR* had a high correlation with the minimum temperature of the coldest month (Figure S10). An extremely high-ranking SNP also occurred upstream of *LIPG*, a gene that regulates lipid levels and in which loss-of-function mutations lead to increased levels of HDL (Edmondson *et al*. 2009). Wolves in especially cold environments may have evolved an increased ability to cope with cold stress by regulating fat metabolism via *LEPR* or *LIPG*. For example, pikas show a significant increase in the rate of non-synonymous substitutions in *LEPR* with lower temperatures (Yang *et al.* 2008), and studies in mice show that *LIPG* may aid in uptake to adipose tissue of free fatty acids (Kratky *et al*. 2003).

We found multiple genes and GO categories related to vision and hearing. One gene identified as a candidate in `BayeScan` and `Bayenv` was *PCDH15*, a member of the cadherin family of proteins that is highly expressed in the retina and cochlea (Alagramam *et al.* 2001). Mutations in this gene have been implicated in Usher Syndrome type 1F, a disease causing deafness (Le Guédard *et al.* 2007). We also found other candidate genes for eye development and hearing, and several related significantly enriched GO categories from the union of all three selection tests (Table S14), and in the `Bayenv` analysis (Table 3, Table S9, Table S10). Wolves inhabit a variety of terrains from open tundra habitats with strong seasonality in light regime to

closed habitat temperate rainforests having more uniform light conditions. Such differences may exert divergent selection pressures on vision and hearing.

Comparison to previous wolf and vertebrate studies

To our knowledge, this study is one of few large-scale genetic analyses of local adaptation in a non-human vertebrate across a substantial range of habitats. We found that precipitation and mean diurnal temperature range were some of the most influential environmental variables associated with SNP variation across the North American range of gray wolves (Figure 2). This result is concordant with previous genetic analysis using microsatellites and mtDNA sequence variation suggesting that vegetation (Geffen *et al.* 2004) and habitat type (Carmichael *et al.* 2007; Muñoz-Fuentes *et al.* 2009) are the main drivers of wolf ecotype differentiation. Precipitation is also a significant correlate of morphological variation in wolves (O'Keefe *et al.* 2013). Consequently, local adaption in wolf ecotypes appears driven by strong environmental gradients, primarily in temperature and precipitation.

Our study provides an advance over previous research by identifying candidate genes in the context of environmental differences among genetically defined ecotypes. Notably, we confirm candidate genes that were outliers in sequencing and SNP genotypes studies of Old World gray wolves suggesting environmental difference may be driving local adaptation there as well. For example, at the 95th percentile cutoff, we observed 173 genes overlapping with a genome sequencing study on high altitude adaptation in Tibetan wolves (Zhang *et al.* 2014) and 14 genes overlapping with a SNP array-based study of demography and outlier SNPs tagging candidate genes in European wolves (Pilot *et al.* 2014) (Figure 7). The two genes common to all three sets, *CACNA2D3* and *AZIN1* are candidates for hypoxia in Zhang, *et al.* (2014). We

speculate that *CACNA2D3* and *AZIN1* may also serve this function in New and Old World wolves given wolf persistence at high and low altitude habitats (Figure 2A).

Functional interpretation of candidate genes under selection in our study was facilitated by a wide array of preexisting studies on pigmentation, disease, and other phenotypes in a variety of species (humans: reviewed in Sturm and Duffy 2012; lab mice: reviewed in Barsh 1996; *Peromyscus* mice: Manceau *et al*. 2011; sheep: Fariello, *et al*. 2014; cattle: Qanbari *et al*. 2014; Arctic skuas: Janssen *et al*. 2013). For example, eight of our candidate genes at the 95th percentile significance overlapped with environmentally correlated genes influencing the "metabolic syndrome" in humans (Hancock *et al.* 2008) (Figure 7). Interestingly, Hancock and colleagues chose to investigate these genes for their involvement in dyslipidemia, obesity, hypertension, type II diabetes, and a "metabolic syndrome" phenotype (see Hancock, *et al.* 2008 for details). The commonality with our study suggests the possibility of a general adaptation toolkit for environmental gradients, such as the *LEPR* gene for cold tolerance, which has also been implicated in cold tolerance and adipose tissue in Neanderthals and Denisovans (Sazzini *et al.* 2014), pikas (Yang *et al.* 2008), and mice (Chua *et al.* 1996). Similarly, we identified common mechanisms of pigmentation and morphology, especially major pathways of bone development such as BMP or WNT. Whereas in humans these genes have been implicated in diseases, selection on these genes in wolves may be a thermoregulatory response to large fluctuations in temperature, osmoregulatory response to differential water availability, or metabolic responses to varying diet and represent local adaptations resulting from divergent natural selection.

Although many phenotypic traits are complex and controlled by multiple genes of small effect (Rockman 2012), we have detected associations of environmental variables and SNPs in

genic regions, which suggests that the effect size is great enough to cause a distortion in allele frequency variation. Our approach for identifying genes involved in adaptation was necessarily correlative and will require further study to confirm whether these candidate genes influence function or are false positives (Barrett & Hoekstra 2011). To determine if tag SNPs are actually associated with potential functional mutations in candidate genes, and if those mutations show evidence of selection (e.g. Domingues *et al.* 2012), new capture array approaches can be used to simultaneously capture exons from thousands of genes followed by high throughput sequencing (Hodges *et al.* 2007). Such verified candidate genes can then be subject to further functional inference or knockout studies to confirm function (e.g. Lewandoski 2001; Storz 2007; Linnen *et al.* 2009; Manceau *et al.* 2011). Nonetheless, the validity of our approach is suggested by previous studies. For example, genes underlying traits shown to be under selection in humans, such as pigmentation, lactase tolerance, and hearing were initially identified as candidates using SNP genotyping (as we have done), and were verified with finer-scale resequencing (reviewed in Akey 2009). Genic SNPs with allele frequencies that follow environmental clines are especially convincing candidates for adaptation. We could observe associations of allele frequency changes with the environment as a result of two patterns: 1) local adaptation of a specific population to distinct environment; or 2) a general clinal trend observed in all populations across an environmental gradient. These patterns are exemplified in Supplemental Figure 10 but unfortunately since we only have six populations, we cannot discriminate statistically between these possibilities.

Two of the methods we used to infer selection (`Bayenv` and `BayeScan`) explicitly control for background demographic patterns, and we conservatively selected the very top few percent of outliers from F_{ST}/XP-EHH, which does not explicitly control for demography.

However, future work incorporating empirically determined demographic models into selection scans and resequencing as discussed above may further clarify the level of false positives (e.g. Freedman *et al.*, in review). Furthermore, resequencing data, which is free from any ascertainment bias and which will more accurately describe variation within populations, is therefore a more sensitive approach to exploring selection. In fact, to further test our conclusions, we have resequenced exons and UTRs for over 1000 candidates genes from over 100 wolves from a similar geographic distribution, as well as 5Mb of non-genic "neutral" sequence. Extensive analyses of these data are described in a companion paper (Schweizer, *et al.*, submitted). We maintain that this general approach, first, using a genome-wide SNP array to identity candidate genes through the use of multiple statistical approaches including environmental data, and second, resequencing candidate genes by genome capture provides an efficient method for documenting and understanding local adaptation in a wide variety of non-model species.

In conclusion, using a SNP genotyping array, we provided evidence for genetic subdivision in North American wolves that correspond to distinct habitats, and consider these populations as unique ecotypes between which divergent natural selection may cause local adaptation despite gene flow. We demonstrated the utility of using multiple selection tests to build an extensive set of candidate genes that may have undergone selection among ecotypes and identify candidate genes for morphology, pigmentation, metabolism, vision and hearing in wolves. Many of these candidate genes also show evidence of local adaptation in Old World wolves and other species. These genes may define a genetic toolkit used by a wide variety of taxa to address climate and environmental variation as well as biotic factors such as food type availability. Our findings demonstrate that despite high mobility we can detect evidence of local

adaptation through a moderately dense genomic scan. This result likely derives from high fidelity to natal habitats of dispersing wolves, strong ecological divergence among habitats, and relatively high levels of linkage in the wolf genome.

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Data Accessibility

Sample locations, environmental data, and SNP genotypes from the Affymetrix SNP array (42K set) are available on the Dryad Digital Repository at datadryad.org (doi:10.5061/dryad.c9b25). Additional files include pairwise geographic and genetic distance matrices used for IBD analysis, the complete Bayenv and Bayescan results files, and the phenotype data used to perform the genotype-phenotype association analysis.

Author Contributions

Designed research: RMS, BVH, RH, JCK, MM, DC, JN, RKW

Performed research: RMS, BVH, RH, JCK

Contributed samples: MM, DC, RKW

Analyzed data: RMS, BVH, RH, JN, RKW

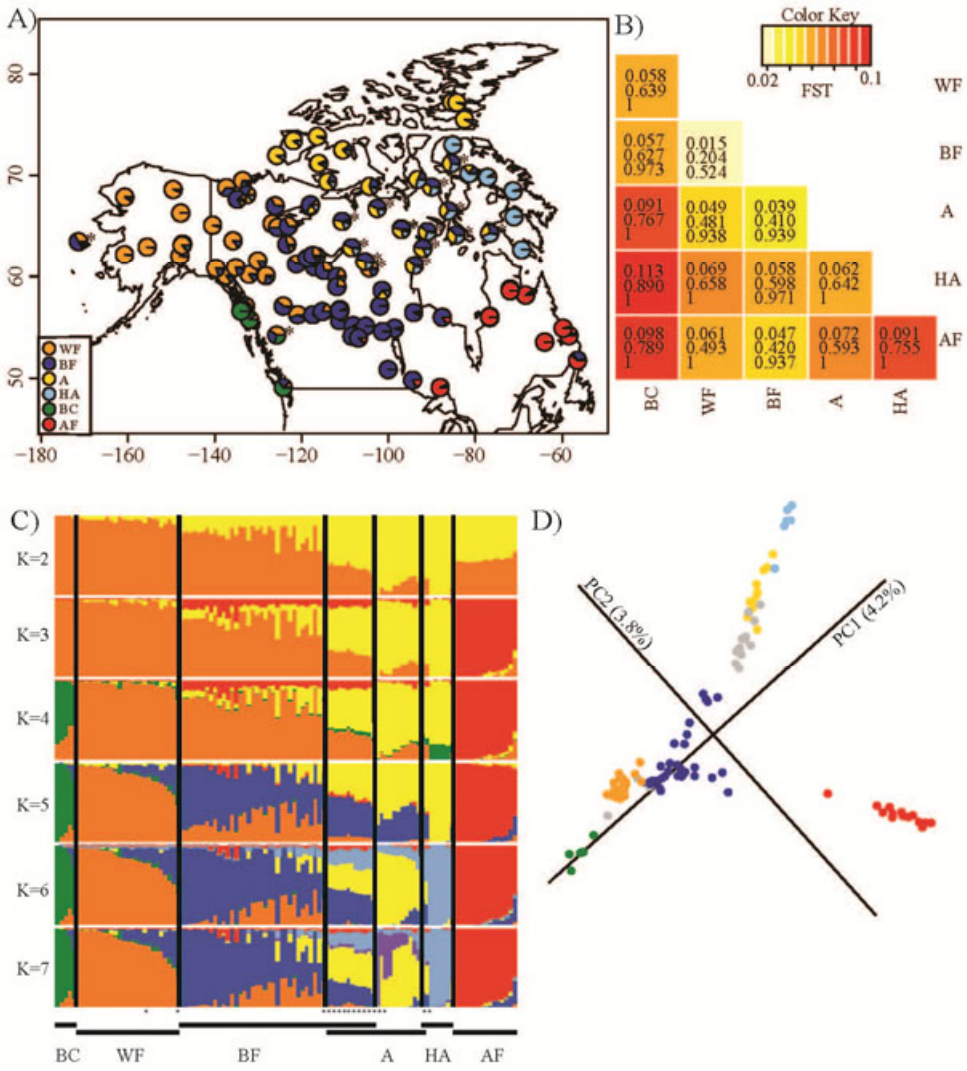
Wrote the paper: RMS, JCK, RKW

Ecotype	General Category	Example(s) of Specific Category	Significance of Specific Category	Type
West Forest	cardiovascular system	Abnormality of the cardiovascular system	2.61E-02	HP
	hearing	functional abnormality of the middle ear	4.01E-02	HP
	membranes	integral component of plasma membrane*	4.18E-02	GO
	metabolism	metabolic pathways	3.63E-02	KEGG
	organismal system	abnormality of the liver	3.67E-02	HP
	skeletal morphology	abnormality of the external nose	1.71E-02	HP
	vision	abnormality of the eye	2.18E-02	HP
Boreal Forest	immune response	immune system process*	2.85E-04	GO
	metabolism	lipid metabolic process*	1.37E-02	GO
	organismal system	tissue development*	2.31E-05	GO
	response to environment	response to external stimulus*	1.14E-04	GO
	skeletal morphology	ossification*	2.29E-04	GO
Arctic	immune response	positive regulation of lymphocyte mediated immunity	4.46E-02	GO
	musculature	abnormality of the musculature	5.00E-02	HP
	organismal system	functional abnormality of bladder	4.71E-02	HP
	skeletal morphology	abnormal bone ossification	4.38E-03	HP
High Arctic	brain function	learning or memory	1.08E-02	GO
	metabolism	histidine metabolism	5.00E-02	KEGG
British Columbia	dentition	misalignment of teeth	4.79E-02	HP
	diet	salivary secretion	4.22E-02	KEGG
	metabolism	Arachidonic acid metabolism	2.73E-03	KEGG
	musculature	Muscle hypertrophy	4.73E-02	HP
	organsismal system	protein transport*	9.31E-03	GO
	skeletal morphology	disproportionate short stature	4.86E-02	HP
	vision	aplasia/hypoplasia of the iris	3.93E-02	HP
Atlantic Forest	dentition	hypodontia	4.20E-02	HP
	metabolism	Glutathione metabolism	5.00E-02	KEGG
	organismal system	calcium ion transmembrane tranporter activity	5.00E-02	GO
	skeletal morphology	aplasia involving forearm bones	4.57E-02	HP

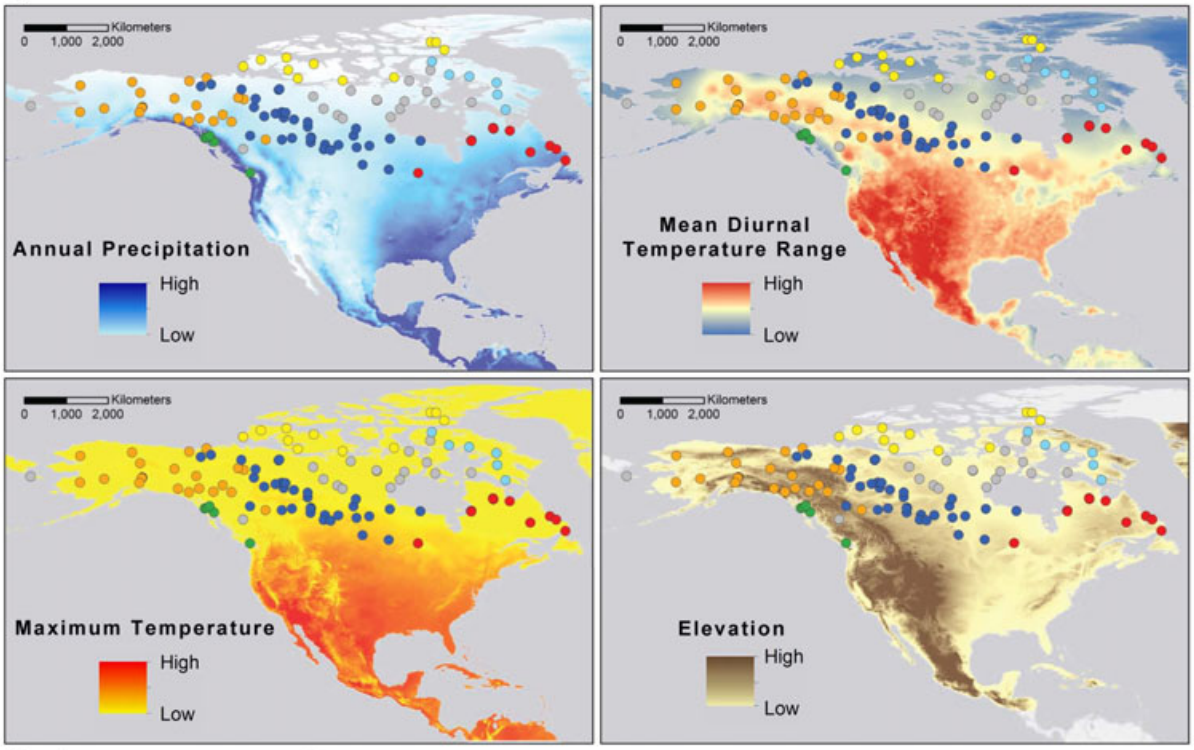
* Indicates category was enriched in top 0.5% candidate genes

General Category	Example of Specific Category	Signifiance	Type
hearing	auditory receptor cell differentiation	5.00E-02	GO
organismal system	Oxytocin signaling pathway	1.02E-02	KEGG
cardiovascular system	Cardiac muscle contraction	2.11E-03	KEGG

General Category	Specific GO Category	TEMPERATURE					PRECIPITATION			VEGETATION			ELEVATION
		BIO1	BIO2	BIO4	BIO5	BIO6	BIO12	BIO15	BIO19	LC	NDVIM	TREE	SRTM
Hearing	ear development/inner ear development								x				
	sensory perception of sound			x*									x
Morphology	anatomical structure development	x	x	x	x	x	x		x	x	x	x	x
	anatomical structure morphogenesis	x		x	x	x	x		x	x	x	x	x
	appendage development				x		x		x			x	
	appendage morphogenesis		x		x		x		x			x	
	growth						x						
	limb morphogenesis		x		x		x		x			x	
	negative regulation of BMP signaling pathway				x								
	positive regulation of ossification		x										
	regulation of growth					x	x		x				
	regulation of muscle tissue development/organ development/cell differentiation				x								
Pigmentation	melanocyte differentiation*		x	x									
	melanin biosynthetic process	x									x	x	
	melanin metabolic process	x										x	
	melanosome membrane											x	
	pigmentation											x	
Smell	olfactory lobe development	x											
System	blood circulation	x								x	x	x	x
	calcium ion binding/calcium ion transmembrane transporter activity	x	x	x		x	x	x	x	x	x	x	x*
	carbohydrate homeostasis						x		x				
	circulatory system process/development	x			x		x		x	x	x	x	x
	developmental growth/process	x	x	x	x	x	x		x	x	x	x	x
	fatty acid transport								x				
	gated channel activity	x			x					x	x	x	x
	lipoprotein particle receptor activity		x										
	locomotion	x	x	x	x	x	x		x	x	x	x	x
	mesenchymal cell differentiation involved in kidney development/renal system		x										
	multicellular organismal response to stress					x	x		x			x	
	negative regulation of ion transport						x						
	passive transmembrane transporter activity		x		x		x			x		x	x
	positive regulation of peptide (hormone) secretion					x	x		x				
	renal system development		x		x	x	x		x				



A)



B)