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DETECTING SELECTION IN NATURAL POPULATIONS: MAKING SENSE OF GENOME SCANS AND TOWARDS ALTERNATIVE SOLUTIONS

Targeted capture and resequencing of 1040 genes reveal environmentally driven functional variation in grey wolves

RENA M. SCHWEIZER,* JACQUELINE ROBINSON,* RYAN HARRIGAN,† PEDRO SILVA,‡ § MARCO GALVERNI,¶ MARCO MUSIANI,** RICHARD E. GREEN,† † JOHN NOVEMBRE‡ ‡ and ROBERT K. WAYNE*

*Department of Ecology and Evolutionary Biology, University of California, Los Angeles, 610 Charles E Young Dr East, Los Angeles, CA 90095, USA, †Center for Tropical Research, Institute of the Environment and Sustainability, University of California, 619 Charles E. Young Drive East, Los Angeles, CA 90095, USA, ‡CIBIO/InBio – Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal, §Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/n., 4169-007 Porto, Portugal, ¶Laboratory of Genetics, ISPRA (Istituto Superiore per la Protezione e Ricerca Ambientale), Via Cà Fornacetta 9, 40064 Ozzano dell'Emilia, BO, Italy, **Faculties of Environmental Design and Veterinary Medicine (Joint Appointment), EVDS, University of Calgary, 2500 University Dr NW, Calgary, Alberta, Canada T2N 1N4 ††Department of Biomolecular Engineering, University of California, Santa Cruz, CA 95060, USA, ‡‡Department of Human Genetics, University of Chicago, 920 E. 58th Street, Chicago, IL 60637, USA*

Abstract

In an era of ever-increasing amounts of whole-genome sequence data for individuals and populations, the utility of traditional single nucleotide polymorphisms (SNPs) array-based genome scans is uncertain. We previously performed a SNP array-based genome scan to identify candidate genes under selection in six distinct grey wolf (*Canis lupus*) ecotypes. Using this information, we designed a targeted capture array for 1040 genes, including all exons and flanking regions, as well as 5000 1-kb nongenic neutral regions, and resequenced these regions in 107 wolves. Selection tests revealed striking patterns of variation within candidate genes relative to noncandidate regions and identified potentially functional variants related to local adaptation. We found 27% and 47% of candidate genes from the previous SNP array study had functional changes that were outliers in SWEED and BAYENV analyses, respectively. This result verifies the use of genomewide SNP surveys to tag genes that contain functional variants between populations. We highlight nonsynonymous variants in *APOB*, *LIPG* and *USH2A* that occur in functional domains of these proteins, and that demonstrate high correlation with precipitation seasonality and vegetation. We find Arctic and High Arctic wolf ecotypes have higher numbers of genes under selection, which highlight their conservation value and heightened threat due to climate change. This study demonstrates that combining genomewide genotyping arrays with large-scale resequencing and environmental data provides a powerful approach to discern candidate functional variants in natural populations.

Keywords: adaptation, capture array, climate, genomics, mammals, natural selection

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Correspondence: Rena M. Schweizer, Fax: 310-206-3987;
E-mail: rschweiz@ucla.edu

Introduction

A principal goal in evolutionary biology is to relate variation in phenotypes to that of underlying genotypes and thus reveal molecular mechanisms for adaptation. The study of organisms in their natural environments is essential in this regard because adaptive phenotypes arise as a result of natural selection. The recent development of genomewide single nucleotide polymorphism (SNP) genotyping technologies has provided new high-resolution tools for exploring adaptation at the molecular level within nonmodel organisms (e.g. Li *et al.* 2014; Perry 2014). Such 'genome scans' are important for identifying regions of the genome tagged by outlier SNPs that may be located within or in linkage disequilibrium (LD) with genes under divergent selection (Haasl & Payseur 2015). However, these molecular studies of adaptation in natural populations may suffer from bias if the SNP genotyping array was not designed for the study species or was based on a narrow ascertainment panel of individuals. Furthermore, the outcome of genomic scans are often merely lists of candidate genes putatively under selection that need to be validated by resequencing to identify potential functional variants that support their role in adaptation (Scheinfeldt & Tishkoff 2013). Advances in the precision and scale of DNA tools, such as the DNA capture array (Hodges *et al.* 2007; Gnirke *et al.* 2009; Tewhey *et al.* 2009; Jones & Good 2015), allow for the enrichment in a DNA sample of specific gene regions for thousands of candidate genes. This targeted enrichment, when followed by high-coverage next-generation sequencing and careful quality control, can be used to confirm signals of selection (Burbano *et al.* 2010; Albert *et al.* 2011; Domingues *et al.* 2012) and pinpoint potential functional mutations (e.g. Ng *et al.* 2009; Bi *et al.* 2013), even in nonmodel organisms (reviewed in Jones & Good 2015). We aim to show that a genome scan followed by extensive resequencing is an effective approach for further verifying putative candidate genes and leads to functional hypothesis about adaptation (Jones & Good 2015). Although direct experimental manipulations to determine function would be ideal, these tests are difficult or impossible in large vertebrates; rather, resequencing in a variety of species including polar bears, kiwis, whitefish and humans, has led to the identification of robust candidates for adaptation (Hebert *et al.* 2013; Fagny *et al.* 2014; Liu *et al.* 2014; Le Duc *et al.* 2015).

The grey wolf (*Canis lupus*) was historically one of the most widespread mammals in North America (Leonard *et al.* 2005), and for a terrestrial mammal, is unique in its ability to disperse long distances >1000 km (Wabakken *et al.* 2007). Despite their high mobility, wolves show striking morphologic and genetic differentiation at a local scale (Carmichael *et al.* 2007; Musiani *et al.* 2007; vonHoldt *et al.* 2011; O'Keefe *et al.* 2013; Pilot *et al.* 2014; Schweizer *et al.* 2015). North American wolves are subjected to strong environmental gradients involving dramatic changes in temperature, precipitation and vegetation from the southern reaches of their geographic range in Mexico to the high Arctic (Geffen *et al.* 2004; Carmichael *et al.* 2007; Musiani *et al.* 2007; Muñoz-Fuentes *et al.* 2009; Schweizer *et al.* 2015). We previously formulated general hypotheses about expected patterns of divergence in genes related to immunity, metabolism, morphology, pigmentation and sensory functions (see Supporting information; Schweizer *et al.* 2015 and references therein) and analysed 42 036 SNPs genotyped on the Affymetrix canid v2 SNP array and environmental data to determine whether we could detect candidate genes for local adaptation (Schweizer *et al.* 2015). We identified six environmentally and genetically distinct wolf ecotypes (or distinct populations): West Forest, Boreal Forest, Arctic, High Arctic, British Columbia and Atlantic Forest. Based on preliminary analysis and results from three complementary selection tests and a review of the current literature, we identified 1040 candidate genes potentially under selection for resequencing using a capture array. This resequencing effort is a necessary second step to identify specific functional variants, affirm they show signals of selection, and assess how well genomewide SNP surveys, such as those used in our previous genome scan, tag genes with nonsynonymous or flanking region variants with divergent allele frequencies among populations.

In this study, we tested the utility of our previous SNP-based genome scan to tag genes under selection by resequencing 980 candidate genes in wolves across an environmental gradient. We assess the fraction of tagged genes which have nonsynonymous mutations or possible regulatory changes in flanking regions and discuss potential functional implications. We supplemented this effort by sequencing 60 additional candidate genes that were not previously tagged by SNPs in the genome scan, but for which existing literature suggests may be involved in local adaptation. We used a custom capture array to resequence these 1040 candidate genes, including their exons and putative regulatory regions, in 107 wolves. With each of three selection tests, we used 5 Mb of nongenic sequence to empirically control for genetic patterns due solely to background demography. We verified that up to 47% of candidate genes from the SNP array selection scan are outliers in the same or similar statistical tests using our sequence data and contain mutations at divergent frequencies across populations. Using available protein

databases, we highlight nonsynonymous mutations in three genes (*APOB*, *LIPG*, *USH2A*) that appear to be under positive selection and occur in functional protein domains. We argue for more conservation focus on Arctic and High Arctic wolves because they demonstrate a high diversity of unique molecular adaptations, yet comprise some of the most threatened populations due to climate change.

Methods

Resequencing of candidate regions with capture array

Our capture array was designed to bind sequences from 1040 candidate genes. Of this total, 520 of the genes were outliers identified in the previous SNP-based selection scan (Schweizer *et al.* 2015), and a total of 60 genes were a priori candidate genes based on a literature search (Table S1, Supporting information). For the latter pool, we chose genes implicated in function or disease that could conceivably be under selection in natural populations, such as genes related to olfaction, immunity, thermoregulation and morphology (Table S1, Supporting information). An additional 460 candidate genes derived from an early analysis of the SNP data were included in the design and manufacture of the capture array in 2011 but were not verified by additional, newer selection tests included in Schweizer *et al.* (2015). This preliminary set was identified using only the XP-EHH selection test (Sabeti *et al.* 2007; vonHoldt *et al.* 2010) and based on the inclusion of additional populations of wolves that were later dropped from the analysis because they did not comprise distinct ecotypes or represented translocated populations. The exons, plus 1000 bp upstream of each gene promoter, were targeted with unique 120-bp RNA baits every 60 bp. We also extracted putatively neutral regions from the dog reference genome (CanFam3.1) to provide background levels of neutral variation for our selection tests. Details of the design of these regions have been described elsewhere (Freedman *et al.* 2014) and follow guidelines set by previous studies in humans (e.g. Wall *et al.* 2008; Gronau *et al.* 2011). Briefly, we identified 1-kb regions that were at least 100 kb from any known or predicted genes, were not within highly repetitive regions of the dog genome, were within uniquely mapping regions of the genome as computed by TALLYMER (Kurtz *et al.* 2008), had phastCons scores <0.5 (Siepel *et al.* 2005) and had GC content within two standard deviations of mean dog genome GC content. The choice of 100 kb between each region was based on observed levels of LD in wolves from the study by Gray *et al.* (2009). A total of 5073 autosomal 1-kb regions were identified. Baits were designed by MYcroarray (Ann Arbor, Michigan) with a total targeted sequence length of ~8 Mb.

Sample selection and library preps

North American wolves were previously sampled in a genomewide selection scan based on SNP array genotyping (N = 111, Schweizer *et al.* 2015). For this study, we re-extracted DNA from 78 of the same individuals for which blood or skin tissue sample remained, and selected an additional 39 individuals from similar geographic areas (Fig. 1; Carmichael *et al.* 2007; Musiani *et al.* 2007). All 117 of these individuals have known geographic locations (Schweizer *et al.* 2015), and 47 have coat colour phenotype information (Musiani *et al.* 2007; Denali National Park Wolf Program).

Genomic DNA was extracted using a Qiagen Mini Prep kit and then sheared to ~250–500 bp using a Bioruptor NGS Sonication System (Diagenode). Sequencing libraries for each individual were prepared using 600–1000 ng of DNA following a with-bead library preparation protocol (Faircloth 2015), and individual samples were labelled with a unique 6-bp index during adapter ligation (Faircloth & Glenn 2012). Individual libraries were target-enriched and PCR-amplified according to the MYbaits protocol (MYcroarray) after a 24-h hybridization and then pooled with 24 individuals per lane. Enriched libraries were 100-bp paired-end-sequenced on an Illumina HiSeq 2000.

Sequence alignment and processing followed the general recommendations of the Broad Institute GATK v2.6-4 'Best Practices' pipeline (https://www.broadinstitute.org/gatk/guide/best-practices; see Supporting information for details on software and parameters). Reads were mapped to the entire reference dog genome (*Canis familiaris*; CanFam3.1) as previous work demonstrates that aligning wolf sequences to this reference produces high-quality genotype calls and minimal reference bias due to ~0.1% sequence divergence between wolves and dogs (Freedman *et al.* 2014). All postmapping processing was done for the set of targeted regions, allowing a 1-kb buffer at either end.

Variant filtering and final sample set

Variants were filtered with GATK VariantFiltration using 10 filter expressions, as recommended by the GATK 'Best Practices' pipeline, as well as depth of coverage ≥10 and minimum genotype quality ≥30. Quality of filtered aligned reads was assessed using the VCFTOOLS package (Danecek *et al.* 2011), and we subsequently required that a site be called in at least 95% of individuals for further analysis. Kinship among individuals was calculated using a LD-pruned set of neutral

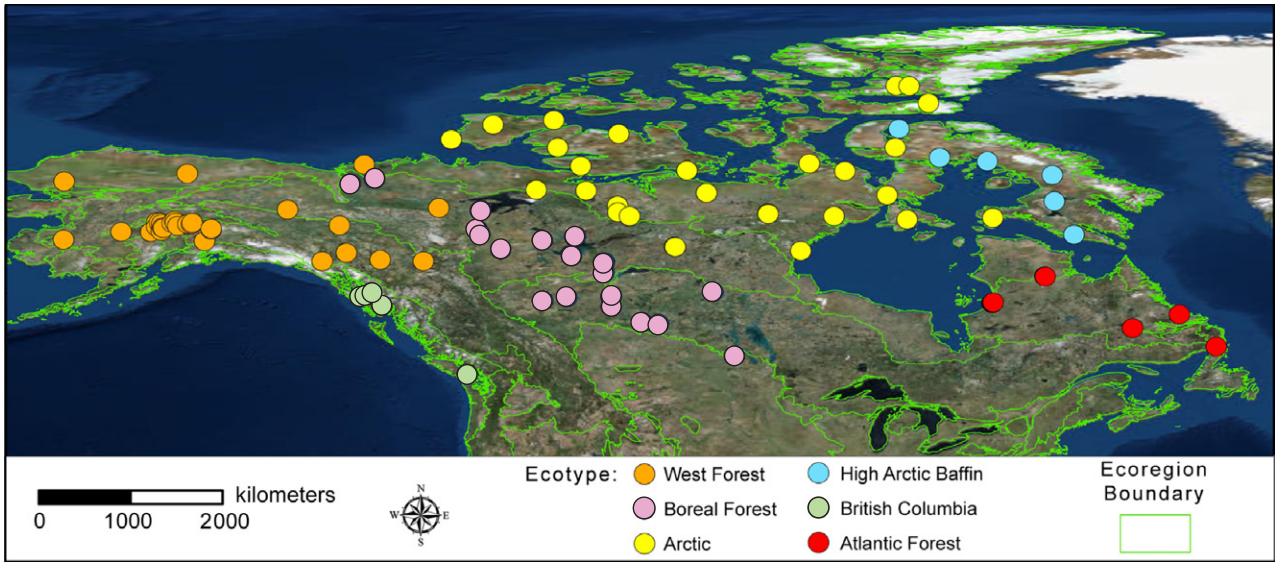

Fig. 1 Sampling location of 107 wolves superimposed on a satellite image, with coloured circles (see legend) indicating the genetically and environmentally determined ecotypes of West Forest, Boreal Forest, Arctic, High Arctic Baffin, British Columbia and Atlantic Forest. Green boundaries show major Environmental Protection Agency Ecoregions (http://www.epa.gov/naaujydh/pages/ecoregions.htm).

variants (using the –indep-pairwise 50 50 0.5 option in PLINK (Purcell *et al.* 2007), as in Schweizer *et al.* (2015) and KING, which accounts for population structure (Manichaikul *et al.* 2010). To remove related individuals, which is commonly done to prevent spurious selection signals caused in part by high identity-by-descent (Anderson *et al.* 2010; Bigham *et al.* 2010; vonHoldt *et al.* 2010; Stranger *et al.* 2011; Fu *et al.* 2012), we used PRIMUS (Staples *et al.* 2012) and a maximum pairwise identity-by-descent of 0.1 (Fu *et al.* 2012).

Ecotype assignment of unrelated individuals was verified by both STRUCTURE v2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003) and ADMIXTURE v1.23 (Alexander *et al.* 2009). Previously, ecotypes have been defined based on ecological and genetic criteria, and are essentially genetically distinct populations that are located in different habitat types (Geffen *et al.* 2004; Carmichael *et al.* 2007; Musiani *et al.* 2007; Schweizer *et al.* 2015). We ran 10 independent runs of STRUCTURE, each with 20 000 burn-in iterations and 50 000 MCMC iterations for $K = 1$ through 10 with correlated allele frequencies under the admixture ancestry model and with no location prior, using a set of 28 195 LD-pruned neutral variants. These parameters are almost identical to those used on similar data sets (vonHoldt *et al.* 2010, 2011; Pilot *et al.* 2014; Schweizer *et al.* 2015), and we monitored alpha and likelihood values for convergence during burn-in and MCMC iterations. The *greedy* algorithm within CLUMPP v 1.1.2 (Jakobsson & Rosenberg 2007) was used to control for variation in cluster labels across the 10 iterations of STRUCTURE. As an alternative clustering method, ADMIXTURE (Alexander *et al.* 2009) was run using the same data set. Ecotype classifications of individuals were based on concordant STRUCTURE or ADMIXTURE assignments. We reasoned that, given different assumptions underlying STRUCTURE and ADMIXTURE, a concordant assignment (>50% for both methods) provides some independent confirmation of groupings. Specifically, STRUCTURE implements a cluster criterion focused on deducing populations in Hardy–Weinberg equilibrium whereas ADMIXTURE is an assumption-free clustering method, and both methods have differing susceptibilities to sample size and the density of geographic localities (Pritchard *et al.* 2000; Alexander *et al.* 2009).

Variant annotation and gene annotation

Functional variants within genic regions were identified and annotated using the dog reference within Ensembl's Variant Effect Predictor (VEP) pipeline (v77) (McLaren *et al.* 2010). The program, Sorting Intolerant From Tolerant (SIFT), which uses sequence alignment conservation across multiple species to identify the potential impact of mutations within coding regions (Ng & Henikoff 2003), was implemented through the VEP software. SIFT scores can be used to rank nonsynonymous mutations as deleterious (score < 0.05) or tolerated (score ≥ 0.05) to indicate putative functional impact. Given that nonsynonymous mutations may have a functional impact even if they do not occur at

highly conserved coding positions, we also used the Miyata score of biochemical similarity (Miyata *et al.* 1979). A Miyata score ≥ 1.85 means the amino acid substitution is significantly different in terms of biochemistry or size, and is an alternative to protein prediction algorithms such as PolyPhen that are designed for humans (Adzhubei *et al.* 2010; Marsden *et al.* accepted).

Putative transcription factor binding sites (TFBS) were identified within genic regions using the profiles in the JASPAR PHYLOFACTS database (http://jaspar.genereg.net/). This database contains count matrices of conserved motifs in human, mouse, rat and dog originally identified by Xie *et al.* (2005). The motifs were converted to probability weight matrices and used with the motif finding program FIMO (Grant *et al.* 2011) (part of the MEME package v4.8.1: http://meme.sdsc.edu) to find matching occurrences in our sequence data.

Genic regions containing nonsynonymous, deleterious nonsynonymous, 5′ untranslated region (UTR), 3′UTR or TFBS mutations were tested for enrichment of Gene Ontology (GO) categories using the R 3.1.3 (http://www.R-project.org) package gProfileR (Reimand *et al.* 2007, 2011), with 'strong' hierarchical filtering and a Benjamini–Hochberg (BH) false discovery rate (FDR) to correct for multiple testing. A list of all genes sampled on the capture array was used as a statistical background for testing enrichment. To determine whether longer genes were more likely to be found as outliers because of increased sampling of variants, we assessed the Pearson correlation of gene length with significance measures from SWEED, and BAYENV, and corrected for multiple tests as above.

Detection of selective sweeps

We applied the site frequency spectrum (SFS)-based method of Nielsen *et al.* (2005), as implemented in the software SWEED (Pavlidis *et al.* 2013). This model detects selective sweeps from genomic SNP data using a composite likelihood-ratio test to choose between neutral or selective sweep models and has the benefit that the null hypothesis is derived from the background pattern in the data itself. By comparing specific allele SFS to the empirical average, the location and magnitude of a selective sweep can be estimated (Nielsen *et al.* 2005).

For each ecotype, we ran SWEED on the data from neutral regions and genic regions separately, using a grid size of 10 000. The *P*-value of each genic position likelihood score was determined by calculating the empirical percentile according to the distribution of likelihood values for the neutral regions, and *P*-value correction for multiple testing was achieved through a BH correction (using the 'ecdf' and 'p.adjust' functions within R). A FDR threshold of 0.05 was used not as a strict threshold, but rather as a parameter to assign especially high support for outliers (e.g. Wenzel & Piertney 2014). With this approach, we aimed to correct for neutral population demographic history without the assumptions of simulating data as there is no prior demographic model available.

To identify the genes nearest each grid position in the output from SWEED, we used BEDTOOLS v2.21.0 (Quinlan & Hall 2010) to intersect the positions with the Ensembl annotation gene set (CanFam3.1, Ensembl v79, March 2015), allowing a 6-kb buffer on either side (N. Alachiotis, personal communication). Only genes that overlapped those queried by the capture array were annotated. We chose a *P*-value ≤ 0.01 cut-off for higher stringency, given recent concern for elevated false positive rates in SWEED under certain scenarios (Crisci *et al.* 2013). Using ANGSD (Nielsen *et al.* 2012), we also generated unfolded SFS to verify patterns of allele frequency variation of the top 5% of genes from SWEED relative to neutral and genic regions (Supporting information).

Diversifying selection

To assess diversifying selection among ecotypes, we used the Bayesian method implemented in BAYESCAN v2.1 (Foll & Gaggiotti 2008). BAYESCAN tests whether the subpopulation-specific allele frequencies are significantly different from those within the common gene pool and computes an alpha value to assess departures from neutrality. Significance is assigned by a measure of support for a model in which selection explains allele frequency differences among populations vs. a null model. Positive alpha values imply diversifying selection and negative values balancing or purifying selection. Given concern for elevated false positive rates for detecting balancing or purifying selection under isolation by distance (Lotterhos & Whitlock 2014), we focus on diversifying selection. In populations where isolation by distance is present, as is the case here (Schweizer *et al.* 2015), BAYESCAN can have high false positive rates unless a large set of neutral loci are used to generate empirical *P*-values (Lotterhos & Whitlock 2014). Therefore, we ran BAYESCAN separately for the neutral and genic regions, with prior odds of 10 000 and 1000, respectively, and calculated empirical *P*-values of alpha with a BH correction for multiple testing, as was done for SWEED.

Environmentally correlated selection

To understand the effect of varying environments on variation in allele frequencies across North American wolf ecotypes, we implemented BAYENV (Coop *et al.* 2010). With BAYENV, we measured the support for a model in which SNPs covary linearly with an environmental variable over a model in which SNPs vary

according to neutral expectation (Coop *et al.* 2010). We randomly picked 10 000 variants from our neutral region SNP set to generate a covariance matrix from the average of every 20 000th iteration over a total of 500 000 iterations. For each genic SNP, the selection mode of BAYENV was run with 100 000 iterations and 12 environmental variables previously shown to be influential in wolf ecotype differentiation (Schweizer *et al.* 2015). The 12 variables were obtained from the WORLDCLIM database (Hijmans *et al.* 2005), normalized as previously described (Coop *et al.* 2010; Schweizer *et al.* 2015), and 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 (percentage tree cover, normalized difference vegetation index, and land cover category) and altitude. The final matrix of Bayes factors (BFs) was obtained by averaging each BF over a total of 10 independent runs to help control for sensitivity of MCMC sampling methods (Coop *et al.* 2010; Blair *et al.* 2014). The same procedure was done for a set of 15 000 random variants from the neutral nongenic sequence to further control for background demographic patterns. We assigned an empirical *P*-value within R to the $\log_{10}$ BF of each genic variant using the neutral distribution. This approach has been shown to reduce falsely elevated BFs that may occur given the pure drift null model inherent within BAYENV (Coop *et al.* 2010; Hancock *et al.* 2010; Chen *et al.* 2012a; Lotterhos & Whitlock 2014). Variants with a BH-corrected FDR ≤ 0.05 were highlighted as having additional support of being selection candidates.

Using the functional consequences of variants and SIFT scores annotated by VEP, we tested for significant excess of functional variants (missense or stop gained), regulatory variants (5′UTR, 3′UTR or splice) or damaging variants (SIFT score < 0.05) in each set of outlier loci. We performed a Fisher's exact test for count data in R using significance thresholds of *P*-value ≤ 0.05, *P*-value ≤ 0.005 and BF ≥ 3 (Kass & Raftery 1995).

Patterns of variation in allele frequency

We also explored genetic and geographic patterns of variation in allele frequency for significant outliers from BAYENV. For variants with an empirical *P*-value ≤ 0.05, we plotted within each ecotype the average allele frequency of the reference nucleotide vs. the average of the significant environmental variable. For the genic SNPs, we also identified the ancestral and derived alleles, when possible, using allelic variation within Israeli wolf, Croatian wolf, Chinese wolf (all *Canis lupus*), and Israeli golden jackal (*Canis aureus*) as an outgroup (Freedman *et al.* 2014).

Overlap assessment between outliers from capture array and SNP array

To gauge the utility of genome scans from SNP arrays in predicting candidates for selection, we examined the overlap of significant outliers between outliers on the capture and the Affymetrix SNP array (Schweizer *et al.* 2015), using only the genes that were assayed with both methods ($n = 739$). We compared overlap within BAYENV, within BAYESCAN, and between SWEED and F_{ST}/XP-EHH. Although the approaches are different between SWEED (SFS-based) and F_{ST}/XP-EHH (haplotype-based) (Sabeti *et al.* 2007), both methods should identify regions containing genes that have been swept to high frequency.

Genotype association with coat colour

Using coat colour information from 47 individuals (eight black, 17 grey, 22 white) from among the West Forest, Boreal Forest and Arctic ecotypes, we tested for significant associations between black or white coat colour and each of the 13 092 genic SNPs (see Results) using the variance component model within EMMAX (Kang *et al.* 2010). The set of LD-pruned neutral SNPs was used to calculate a Balding–Nichols kinship matrix, and genic SNPs were pruned for minor allele frequency ≥10% (Kang *et al.* 2010). Multiple testing *P*-value correction was performed within R using the 'p.adjust' function.

Protein models

For candidate genes that had publicly available protein structure information, we explored the effect of functional variants on structure. We extracted the coding sequence from the reference dog genome using the HTSeq (Anders *et al.* 2015) and BIOPYTHON (Cock *et al.* 2009) modules within PYTHON v2.6 (www.python.org), translated the sequence to amino acids using phase information from Ensembl with *ExPASy* (Gasteiger 2003), and then aligned the protein sequences to human annotated versions within GENEIOUS v8.1.3 (Kearse *et al.* 2012) to identify allelic variation. We modelled functional impact on three-dimensional protein structure using SWISS-MODEL (Arnold *et al.* 2006).

Results

Resequencing of candidate regions with capture array

The overall sequencing quality was high, with per-individual average unfiltered yield of 1889.83 ± 567.42 Mb,

88.91 ± 3.52% raw reads passing Illumina filters, and mean quality of 34.5 ± 0.88. After processing and removing low-quality reads, 89 ± 14% raw reads mapped to the dog reference genome and 86 ± 6% of raw reads mapped uniquely to the dog reference genome (i.e. after PCR duplicate removal). After genotyping and additional filtering, the mean depth of coverage over all regions on the capture array was 154.78× ± 64.45×, with mean neutral coverage of 181.65× ± 72.95× and mean genic coverage 89.61× ± 31.22× (Fig. S1, Supporting information). After filtering, we identified 4 918 729 neutral positions and 2 129 544 genic positions, of which 39 376 and 13 092 were variable, respectively (Table S2, Supporting information). The transition to transversion rates were 2.32 for neutral regions and 4.17 for exonic regions (Table S2, Supporting information), which are similar to values in wolves and humans (DePristo *et al.* 2011; Freedman *et al.* 2014; Zhang *et al.* 2014). Genotype quality was assessed by comparing genotypes for 198 SNP positions overlapping with the Affymetrix SNP array and the capture array target regions in the same 78 individuals (Schweizer *et al.* 2015). Genotyping concordance was >99.5% (Table S3, Supporting information).

After removal of two related individuals, ecotype assignment was confirmed for all individuals by concordant assignment in STRUCTURE and ADMIXTURE. Eight individuals were removed from further analyses as neither STRUCTURE nor ADMIXTURE could assign them to a single ecotype with >50% assignment, and our selection tests required that each individual be assigned to a population. The remaining set of 107 individuals included 31 West Forest, 26 Boreal Forest, 30 Arctic, six High Arctic, five British Columbia and nine Atlantic Forest wolves (Fig. 1).

Variant annotation and GO enrichment

VEP annotated a total of 13 092 variants (Table S2, Supporting information). GO enrichment analysis of genes containing functional variants (missense, deleterious missense, 5′UTR, 3′UTR and TFBS) identified 80, 30, 50, 280 and 113 significantly enriched categories, respectively (BH-corrected P-value ≤ 0.05). We focused on GO categories with a minimum of five genes at the highest hierarchical level and found that four of 31 categories overlapped between functional variant category types (Fig. S2, Supporting information). The most significantly enriched GO category was 'detection of chemical stimulus involved in sensory perception' (P-value: 3.92e-06) in missense variants, with the next two most significant categories in related categories of 'olfactory receptor activity' and 'detection of stimulus involved in sensory perception'. Within human phenotype categories, we identified 17 categories with a minimum of five genes, but no significant categories in 5′UTR variants (Fig. S3, Supporting information).

Detection of selective sweeps

Using SWEED, we identified candidate selective sweep regions putatively under selection in each wolf ecotype (Figs 2 and S4–S9, Supporting information). Tests for GO enrichment of significant outliers (P-value ≤ 0.01), with a minimum of two genes in each category, identified four significant categories at the highest hierarchy. 'Defence response' was enriched in High Arctic wolves (P-value: 0.05), and cellular-related categories were enriched in Arctic, Atlantic Forest and British Columbia wolves (Fig. S10, Supporting information). Human phenotype categories demonstrated enrichment of genes related to 'round face' and 'short neck' in Boreal Forest wolves, and 'infantile onset' and 'aplasia/hypoplasia involving the central nervous system' in Arctic wolves (Fig. S11, Supporting information). Arctic and High Arctic wolves had the highest numbers of candidate genes at this threshold and the highest number of significantly enriched GO-related categories (Fig. 2A), as well as the highest numbers of unique candidate genes (Fig. 2B). Furthermore, Arctic and High Arctic ecotypes had the highest number of microRNA categories (Fig. 2A), and showed a high proportion of low-frequency and high-frequency derived alleles, relative to neutral regions (Fig. S12, Supporting information). Within each of the six ecotypes, the Pearson correlation between gene length and maximum-likelihood value within each gene was low (−0.025 to 0.211), but was significant for four populations (West Forest, Boreal Forest, Arctic, Atlantic Forest; Table S4, Supporting information). These findings suggest a lack of power to detect selection in short genes, in some cases.

Within SWEED results, we focused on significant missense variant positions (P-value ≤ 0.05), as the functional effects are more directly interpretable, although many more variant types were identified in significant genes (Figs S4–S9, Supporting information). We identified 25 genes (57 missense variants) in West Forest wolves, 29 genes (77 missense variants) in Boreal Forest wolves, 34 genes (112 missense variants) in Arctic wolves, 24 genes (78 missense variants) in High Arctic wolves, 25 genes (101 missense variants) in British Columbia wolves, and 34 genes (96 missense variants) in Atlantic Forest wolves. It is of interest to note that significant variants in putative TFBS were within sweep regions in all populations (West Forest: 20 genes; Boreal Forest: 21 genes; Arctic: 22 genes; High Arctic: 16 genes; British Columbia: 21 genes; Atlantic Forest: 20 genes).

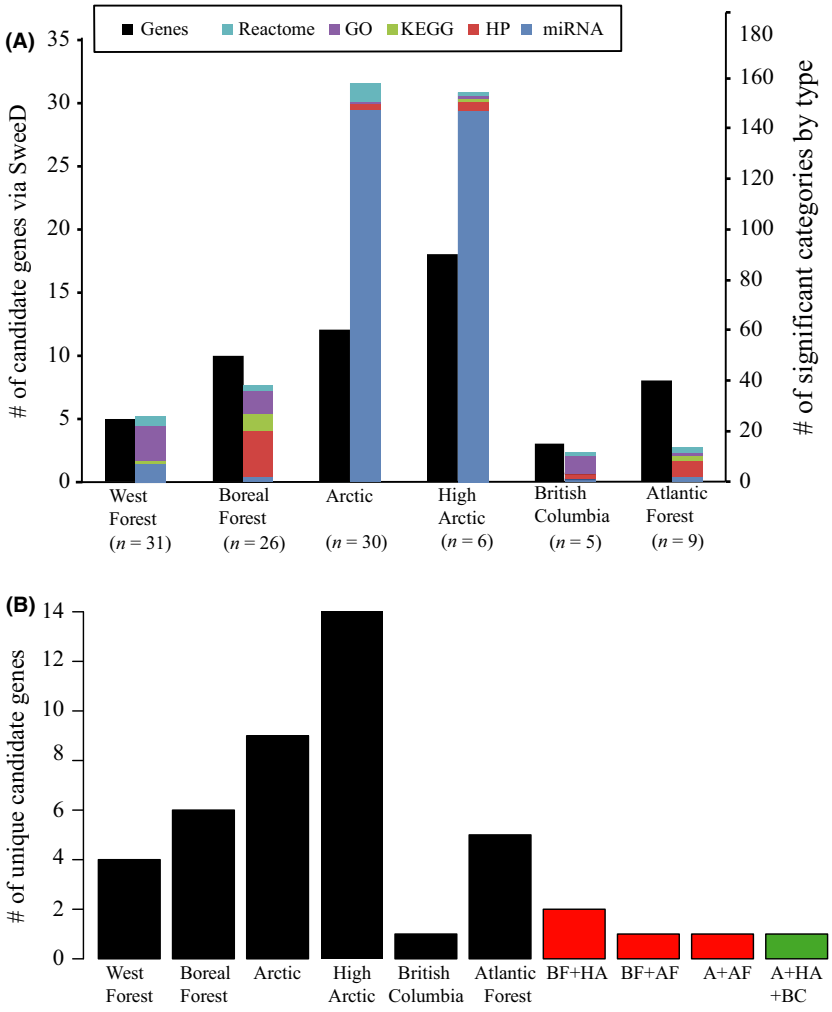

Fig. 2 Counts of genes and GO-related categories from SWEED. (A) The number of candidate genes and GO-related categories within wolf ecotypes (*n*: sample size), using a significance threshold of $P \leq 0.01$. Reactome: Reactome Biological Pathway; KEGG: Kyoto Encyclopedia of Genes and Genomes Pathway; GO: Gene Ontology; HP: Human Phenotype; miRNA: miRBase microRNAs. (B) The number of unique candidate genes at the same significance threshold within each ecotype and within more than ecotype. Ecotypes are coded as follows: WF (West Forest), BF (Boreal Forest), A (Arctic), HA (High Arctic), BC (British Columbia), AF (Atlantic Forest).

There were several notable outlier genes from SWEED. *APOB* (*Apolipoprotein B*), which controls plasma cholesterol levels in a wide range of species (Farese *et al.* 1995), was an outlier in British Columbia wolves (Fig. S8, Supporting information). In Arctic wolves, a selective sweep region was centred on a candidate gene for hearing and vision, *PCDH15* (*Protocadherin 15*; Alagramam *et al.* 2001; Le Guédard *et al.* 2007) (Fig. S6, Supporting information). In both Arctic and Atlantic Forest wolves (Figs S6 and S9, Supporting information), the olfactory receptor gene *OR6B1* was a significant outlier with missense mutations. Two immunity-related canine beta-defensins, *CBD102* and *CBD1*, were highly ranked in West Forest and High Arctic wolves, respectively (Figs S4 and S7, Supporting information). The immunity-related MHC class II gene, *DLA-DQA* (Wagner *et al.* 1996), was also highly ranked in Atlantic Forest wolves (Fig. S9, Supporting information). *TYR* (*Tyrosinase*), which encodes the rate-limiting enzyme that converts tyrosine to melanin within the pigmentation pathway, was an outlier in Boreal Forest and Arctic wolves (Figs 6 and S5, Supporting information). *TYRP1* (*tyrosinase-related protein 1*), which was an outlier within British Columbia wolves (Fig. S8, Supporting information), can cause brown or white colour in dogs and mice (Nakamura *et al.* 2002; Kaelin & Barsh 2013). *MLPH* (*melanophilin*) was an outlier in Atlantic Forest wolves, with a *P*-value < 0.01 (Fig. S9, Supporting information), and mutations within *MLPH* have been associated with the 'dilution' phenotype, in which eumelanin pigment appears diluted to silver colours (Hume *et al.* 2006).

Diversifying selection

Our analysis with BAYESCAN identified three SNPs with a BH-corrected *P*-value ≤ 0.05. One significant SNP (α: 1.42) causes a synonymous amino acid change in *UACA* (*uveal autoantigen with coiled-coil domains and ankyrin repeats*), a gene that regulates apoptosis in response to

stress, and has been implicated in multiple vision-related disorders (Yamada *et al.* 2001; Ohkura *et al.* 2004). Another significant SNP (α: 1.52) is located in an intron of *ATP10B* (*ATPase, Class V, Type 10B*), a gene that is involved in phospholipid translocating and significantly associated with coronary artery disease and degree of atherosclerosis (Nolan *et al.* 2012). The remaining significant SNP was intergenic (α: 1.37). GO enrichment of the two genes did not identify any significant categories with more than one gene overlap.

Environmentally correlated selection

Using the BAYENV method, we focused on the effect of 12 environmental variables on missense and TFBS variants. Only deleterious missense variants (i.e. with SIFT score ≤ 0.05 and *P*-value ≤ 0.005 or BF ≥ 3) were enriched in temperature seasonality and precipitation seasonality. We did not find significant enrichment of broader functional or regulatory mutations in any other environmental variables. GO analysis of genes with significant variants (*P*-value ≤ 0.005) in BAYENV identified significant enrichment in multiple ecologically relevant top-level categories, including those related to vision, hearing, immunity and homoeostasis (Fig. 3). There was overlap of GO categories among similar types of environmental variables (i.e. vegetation, precipitation or temperature; Fig. 3). Human phenotype category enrichment of the same set of genes revealed categories for hearing, vision and bone development (Fig. S13, Supporting information). The Pearson correlation between gene length and maximum BF was significant for two variables (minimum temperature of coldest month, precipitation seasonality; Table S4, Supporting information).

Several missense mutations were highly and significantly correlated with environmental variables (Fig. 4, Table 1). These mutations occurred within genes that function in olfactory receptor activity, coat coloration, lipid metabolism, vision and hearing, and immunity. Detailed statistics and specific genes are provided in Table 1, and patterns of allele frequency variation for a subset are shown in Figs 4 and 5. We note that we did not find any missense mutations in *CBD103*, but this was not expected given previous studies showing that a 3-bp deletion causes black coat colour (Candille *et al.* 2007). We did find that an intron variant within 596 bp of the 3-bp deletion that was in perfect linkage ($D' = 1$; Fig. 4)

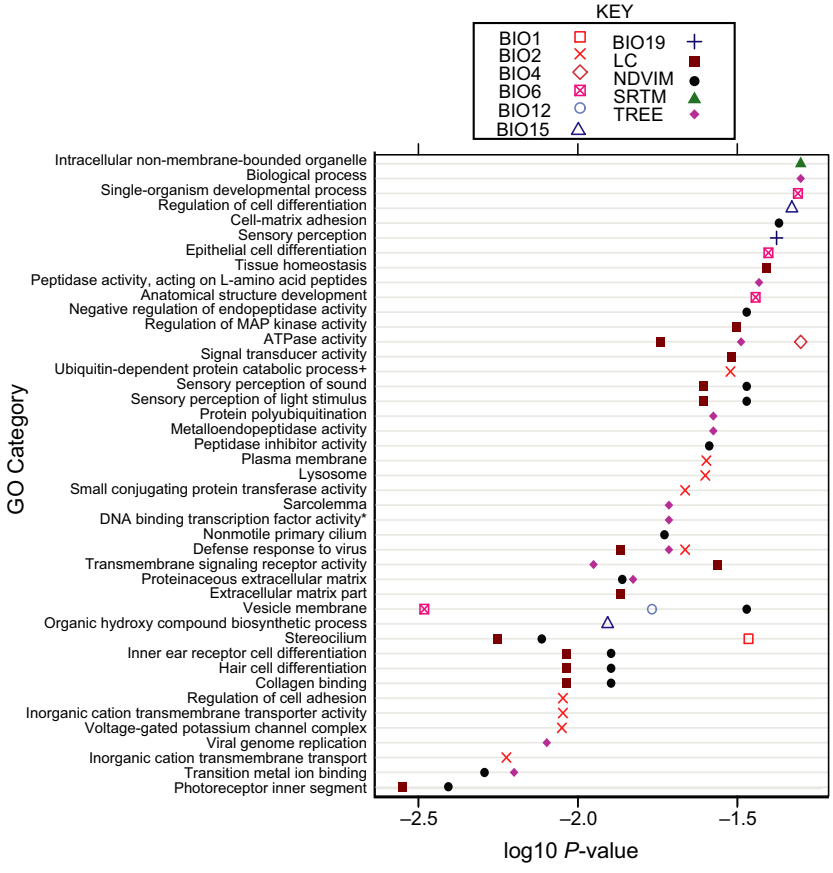

Fig. 3 Significantly enriched Gene Ontology (GO) categories containing genes with mutations significant in BAYENV ($P < 0.005$). Only categories with a minimum of two genes are shown, with the log10 *P*-value as calculated by gProfileR and significant after multiple testing. Two categories shortened for space limitation are marked: 'positive regulation of sequence-specific DNA binding transcription factor activity' (*) and 'protein ubiquination involved in ubiquitin-dependent protein catabolic process' (+). Environmental variables are related to temperature (red colours; BIO1: annual mean temp., BIO2: mean diurnal temp. range, BIO4: temp. seasonality, BIO5: max. temp. of warmest month, BIO6: min. temp. of coldest month), precipitation (blue colours; BIO12: annual precipitation, BIO15: precipitation seasonality, BIO19: precipitation of coldest quarter), vegetation (green colours; LC: land cover metric, NDVIM: normalized difference vegetation index, TREE: percentage tree cover) and elevation (black; SRTM: shuttle radar topography metric).

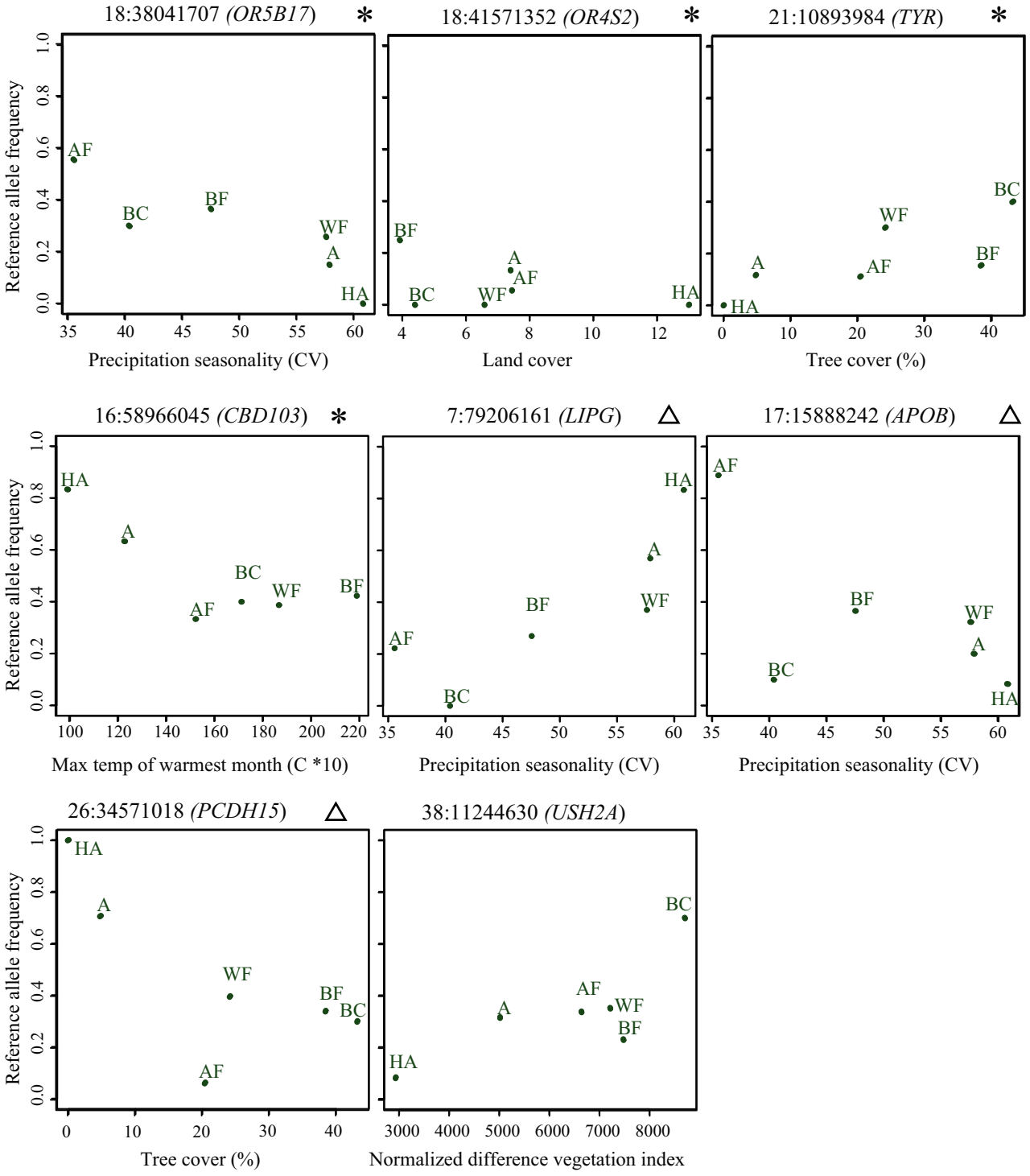

Fig. 4 Variation in allele frequency for eight significant variants from BAYENV. For each single nucleotide polymorphism (SNP), the reference allele frequency (*y*-axis) and environmental variable (*x*-axis) are plotted. The gene name and SNP chromosome and base pair position (in CanFam3.1) are provided, with an indication that the derived allele is reference (Δ) or nonreference (*), if known. Ecotypes are coded as follows: WF (West Forest), BF (Boreal Forest), A (Arctic), HA (High Arctic), BC (British Columbia), AF (Atlantic Forest).

and was significantly associated with maximum temperature of warmest month (BF = 4.2; *P*-value = 0.0037) and land cover type (BF = 2; *P*-value = 0.0076).

We also examined whether any significant noncoding variants from BAYENV were located in putative TFBS, and found six variants (*P*-value ≤ 0.005) overlapping six

Table 1 Summary table of significant nonsynonymous SNPs identified in BAYENV

Gene	SNP	Environmental variable		Nucleotide mutation	Ancestral	Amino acid mutation	SIFT score	Miyata score	Bayes factor	*P*-value
APOB (lipid metabolism)	17:15881156	BIO15	Precipitation seasonality	C/T	?	Arg3812Lys	1	0.4	4.07	0.00293
	17:15881156	TREE	Percentage tree cover	C/T	?	Arg3812Lys	1	0.4	2.26	0.00853
	17:15881156	SRTM	Altitude	C/T	?	Arg3812Lys	1	0.4	1.89	0.02400
	17:15882467	SRTM	Altitude	C/T	C	Val3510Ile	1	0.85	1.23	0.04413
	17:15884825	BIO15	Precipitation seasonality	C/T	C	Val2724Ile	0.89	0.85	2.56	0.00560
	17:15884825	BIO5	Max. temp. of warmest month	C/T	C	Val2724Ile	0.89	0.85	1.57	0.01107
	17:15884825	BIO2	Mean diurnal temp. range	C/T	C	Val2724Ile	0.89	0.85	1.15	0.01573
	17:15886439	SRTM	Altitude	G/T	G	Leu2186Ile	0.06	0.14	13.10	0.00267
	17:15888047	SRTM	Altitude	C/T	C	Gly1650Ser	0.45	0.85	2.27	0.01833
	17:15888047	BIO5	Max. temp. of warmest month	C/T	C	Gly1650Ser	0.45	0.85	1.13	0.01813
	17:15888117	NDVIM	Vegetation index	C/T	?	Met1626Ile	0.27	0.29	1.96	0.00793
	17:15888242	BIO15	Precipitation seasonality	G/A	A	Leu1585Phe	0.32	0.63	7.86	0.00160
	17:15888242	BIO1	Annual mean temp.	G/A	A	Leu1585Phe	0.32	0.63	2.89	0.00360
DLA-DQA (immunity)	12:2221262	TREE	Percentage tree cover	G/C	G	Glu25Asp	0.8	0.9	1.05	0.02167
	12:2225320	SRTM	Altitude	A/C	C	Met99Leu	0.64	0.41	2.77	0.01467
	12:2225320	BIO5	Max. temp. of warmest month	A/C	C	Met99Leu	0.64	0.41	1.19	0.01660
	12:2225320	TREE	Percentage tree cover	A/C	C	Met99Leu	0.64	0.41	1.11	0.02033
	12:2225338	BIO2	Mean diurnal temp. range	A/C	C	Lys105Gln	0.28	1.06	164.00	0.00000
	12:2225338	BIO5	Max. temp. of warmest month	A/C	C	Lys105Gln	0.28	1.06	6.58	0.00213
	12:2225338	SRTM	Altitude	A/C	C	Lys105Gln	0.28	1.06	1.92	0.02307
DLA-DRB1 (immunity)	**12:2164457**	SRTM	Altitude	G/A	?	Pro36Ser	0.72	0.56	2.31	0.01820
LIPG (lipid metabolism)	7:79206161	BIO2	Mean diurnal temp. range	A/G	G	Ile420Thr	0.43	**2.14**	1.58	0.01027
	7:79206161	BIO15	Precipitation seasonality	A/G	G	Ile420Thr	0.43	**2.14**	1.01	0.01767
OR4S2 (olfaction)	18:41571000	BIO15	Precipitation seasonality	T/C	T	Tyr82His	**0**	**2.27**	1.01	0.01767
	18:41571136	BIO4	Temp. seasonality	G/A	G	Arg127His	1	0.82	1.19	0.01173
	18:41571261	SRTM	Altitude	C/A	C	Leu169Ile	0.13	0.14	1.30	0.04107
	18:41571352	LC	Land cover type	G/A	G	Ser199Asn	**0.01**	1.31	7.34	0.00173
	18:41571352	BIO5	Max. temp. of warmest month	G/A	G	Ser199Asn	**0.01**	1.31	1.75	0.00987

Table 1 *Continued*

Gene	SNP	Environmental variable		Nucleotide mutation	Ancestral	Amino acid mutation	SIFT score	Miyata score	Bayes factor	*P*-value
	18:41571352	NDVIM	Vegetation index	G/A	G	Ser199Asn	**0.01**	1.31	1.03	0.01873
OR5B17 (olfaction)	**18:38041707**	BIO15	Precipitation seasonality	C/T	C	Ala97Val	0.06	1.85	2.26	0.00593
	18:38041775	BIO5	Max. temp. of warmest month	T/C	?	Cys120Arg	1	**3.06**	1.28	0.01480
OR6B1 (olfaction)	16:5885672	NDVIM	Vegetation index	C/G	C	Val48Leu	1	0.91	15.20	0.00073
PCDH15 (vision and hearing)	**26:34571018**	TREE	Percentage tree cover	A/G	G	Asn1555Asp	0.08	0.65	1.04	0.02207
	26:34571630	TREE	Percentage tree cover	G/A	?	Glu1755Lys	1	1.14	1.07	0.02127
TYR (pigmentation)	21:10893984	SRTM	Altitude	C/T	?	Val59Ile	0.31	0.85	1.39	0.03707
	21:10893984	TREE	Percentage tree cover	C/T	?	Val59Ile	0.31	0.85	1.06	0.02140
TYRP1 (pigmentation)	11:33329087	BIO6	Min. temp. of coldest month	G/A	G	Arg416Lys	0.39	0.4	2.81	0.00220
	11:33329087	BIO12	Annual precipitation	G/A	G	Arg416Lys	0.39	0.4	1.18	0.00447
USH2A (vision and hearing)	**38:11244630**	NDVIM	Vegetation index	C/T	?	Ala3218Thr	0.58	0.9	5.00	0.00273
	38:11244630	LC	Land cover type	C/T	?	Ala3218Thr	0.58	0.9	1.30	0.01207
	38:11244661	BIO19	Precipitation of coldest quarter	T/G	T	Gln3207His	0.33	0.32	3.25	0.00173
	38:11244661	BIO12	Annual precipitation	T/G	T	Gln3207His	0.33	0.32	2.57	0.00213
	38:11244661	BIO4	Temp. seasonality	T/G	T	Gln3207His	0.33	0.32	2.47	0.00527
	38:11244661	BIO6	Min. temp. of coldest month	T/G	T	Gln3207His	0.33	0.32	1.55	0.00440
	38:11288551	SRTM	Altitude	C/T	C	Asp2828Asn	1	0.65	21.70	0.00167
	38:11297838	BIO5	Max. temp. of warmest month	G/A	A	Ala2692Val	0.33	**1.85**	1.10	0.01867

Single nucleotide polymorphisms (SNPs) with allele frequencies plotted against environmental variables in Figs 4 and 5 are in bold. The genotype, reference and nonreference allele of outlier samples (Freedman *et al.* 2014), as well as ancestral allele are provided, when possible (otherwise indicated with '?'). For each SNP, Sorting Intolerant From Tolerant (SIFT) scores ≤ 0.05 and Miyata scores ≥ 1.85 are in bold. See text for details.

genes (Table 2). Notably, we identified a high-ranking variant within a TFBS 567 bp upstream of *LEP* (*leptin*), a gene that encodes a protein secreted from adipose tissue that is involved in obesity (Mammès *et al.* 1998). In humans, a 5′ variant located 633 bp upstream significantly associates with obesity (Li *et al.* 1999). A second variant was located in a putative TFBS for *FOXA3* (*forkhead box A3*) (Table 2). *FOXA3* is itself a transcription factor hypothesized to control expression of multiple liver-related genes and differentiation of adipocytes (Xu *et al.* 2013) and glucose homoeostasis (Shen 2001).

Patterns of variation in allele frequency

Outlier genic SNPs from BAYENV showed large allele frequency differences across environmental variables (Figs 4 and 5, Table 1). Often, the High Arctic and British Columbia ecotypes defined opposite extremes of

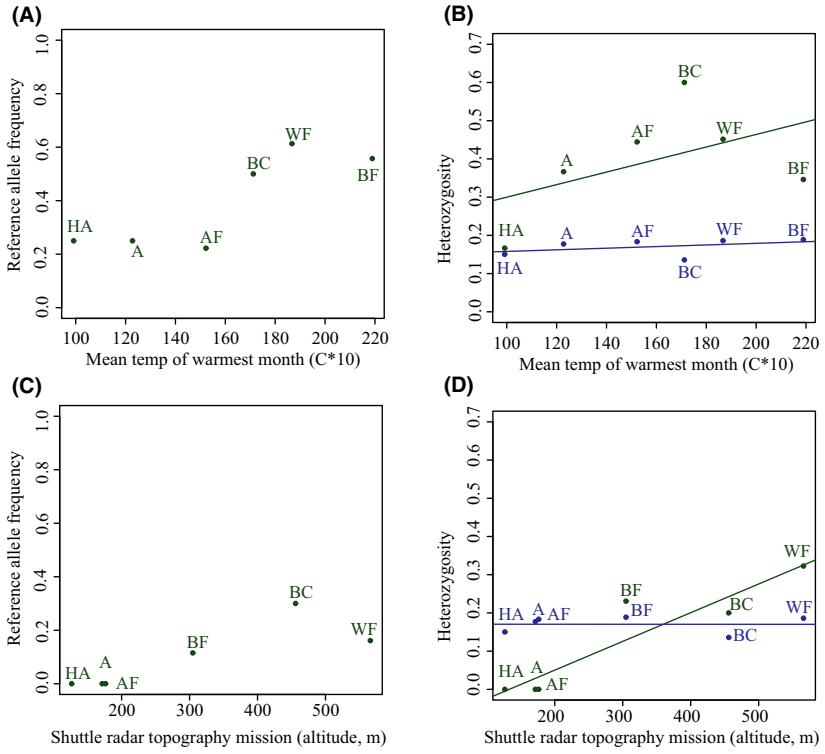

Fig. 5 Allele frequency and heterozygosity in MHC Class II genes. (A) and (B) correspond to *DLA-DQA* missense mutation (chr12:2225338; derived allele is reference), (C) and (D) correspond to *DLA-DRB1* missense mutation (chr12:2164457; ancestral state unknown). The single nucleotide polymorphism (SNP) location and gene name are provided with linear best fit lines and Pearson's correlation ('corr'). Heterozygosity of random neutral SNPs is provided (blue). Ecotypes are coded as follows: WF (West Forest), BF (Boreal Forest), A (Arctic), HA (High Arctic), BC (British Columbia), AF (Atlantic Forest).

Table 2 Summary table of significant single nucleotide polymorphisms (SNPs) from BAYENV that are located in transcription factor binding sites. For each SNP, chromosome and base pair position is provided in CanFam3.1 coordinates, with the nucleotide mutation (reference/nonreference). 'Consequence' refers to the functional impact predicted by Variant Effect Predictor (McLaren *et al.* 2010)

Gene	SNP	Environmental variable		Consequence	Nucleotide mutation	Bayes factor	*P*-value
ATP6V1C2	17:7601977	BIO2	Mean diurnal temp. range	5′UTR variant	C/A	15.00	0.000933
COBLL1	36:9958211	BIO6	Min. temp. of coldest month	Intron variant	C/T	6.26	0.001333
	36:9958211	BIO4	Temp. seasonality	Intron variant	C/T	4.56	0.002800
	36:9958211	BIO19	Precipitation of coldest quarter	Intron variant	C/T	3.83	0.001533
	36:9958211	BIO12	Annual precipitation	Intron variant	C/T	3.28	0.001667
FOXA3	1:109757833	TREE	Percentage tree cover	Upstream gene variant	T/A	4.29	0.004000
GPR116	12:15071633	BIO5	Max. temp. of warmest month	Intron variant	C/T	5.67	0.002400
KLF12	22:27986043	BIO15	Precipitation seasonality	Intron variant	G/A	3.14	0.004000
LEP	14:8121117	TREE	Percentage tree cover	Intron variant	A/G	10.70	0.001400
	14:8121117	LC	Land cover type	Intron variant	A/G	5.77	0.002267

both the environmental variable and SNP allele frequency (Fig. 4). In contrast, we found that often the Boreal Forest and West Forest ecotypes have intermediate allele frequencies and environments (Fig. 4). For 19 outlier SNPs, we were able to infer the ancestral and derived alleles by comparing to previously sequenced wolf and golden jackal genomes (Table 1). For *LIPG*, *OR5B17*, *OR4S2*, *PCDH15* and *TYR*, the Arctic and High Arctic ecotypes show an increase in derived allele frequency, with the greatest change occurring in *PCDH15* where Atlantic Forest wolves were almost fixed for the ancestral allele, and High Arctic wolves were fixed for the derived allele (Fig. 4). In *APOB*, *DLA-DQA*, *OR4S2* and *OR6B1*, we found novel variants not previously observed in Old World wolves and a golden jackal (Freedman *et al.* 2014).

Selection test overlap

We found relatively high overlap between significant genes with a *P*-value ≤ 0.05 for SWEED, BAYESCAN and BAYENV (Fig. S14, Supporting information). Of a total of

554 genes, 195 genes (35.4%) were common to two of three methods and one gene was common to all three methods (*ATP10B*). For the former category, the majority (194/195) of genes overlapped between SWEED and BAYENV. Nineteen of our 60 a priori candidates were significant (P-value ≤ 0.05) in at least one selection test. Using a stricter threshold (SWEED P-value ≤ 0.01, BAYESCAN P-value ≤ 0.01, BAYENV BF ≥3), there were no genes common to all three methods (Fig. S14, Supporting information). There were, however, 28/233 genes (12.0%) common to BAYENV and SWEED, and 3/233 genes (1.3%) common to BAYENV and BAYESCAN. The 28 genes common to BAYENV and SWEED at this threshold included five candidate genes mentioned above: *CBD1*, *CBD102*, *CBD103*, *MLPH* and *PCDH15*.

Overlap between capture array and SNP array

To determine how well our previous selection scan identified candidate genes (Schweizer *et al.* 2015), we assessed the overlap between candidate genes tagged by the SNP array and sequenced by capture array. There were a total of 739 genes on the capture array that were within 10 kb of a SNP on the Affymetrix dog SNP array. BAYENV performed the best, with 188/296 genes (47%) occurring in the top 5% rank in both platforms (Fig. S15, Supporting information). Selective sweep methods (SWEED and F_{ST}/XP-EHH) also showed concordance, with 73/270 (27%) genes overlapping, even though the analytical methods differed between the SNP array and the capture array (Fig. S15, Supporting information). No outlier genes from BAYESCAN on the SNP array were confirmed by gene sequencing on the capture array (out of six overlapping). We also observed cases in all three selection methods using resequencing data where the test identified significant genes that had been tagged by SNPs on the Affymetrix array but were not identified within the top 5% of genes (or FDR < 0.05 for BAYESCAN) on the Affymetrix array (BAYENV: 172 genes; BAYESCAN: two genes; sweeps: 115 genes).

Genotype association with coat colour

Using data from eight black, 17 grey and 22 white wolves, we found significant associations with SNPs in pigmentation genes. In black wolves, eight SNPs had a corrected q-value ≤ 0.05, and the most significant SNP in a pigmentation gene was a 3′UTR variant in *CBD103* (q-value: 0.02895). The other seven SNPs were within the selective sweep region for *CBD103* (Anderson *et al.* 2009). In white wolves, there were three significant SNPs (corrected q-value ≤ 0.05), all within the 3′UTR region of *CBD103* (most significant q-value: 0.04467).

Protein models

To further explore the potential impact of selected variants on protein function, we chose three high-ranking genes, *APOB*, *LIPG* and *USH2A*, for which protein domain structure and other relevant literature were readily available. For *APOB*, which is one of the most complex proteins in the genome with regard to exon structure, we focused on exon 26, which encodes the most important functional domains (Young 1990; Amrine-Madsen *et al.* 2003). Three missense mutations within *APOB* occurred in a region from AA 1425 to AA 1728 in humans (AA 1563 to AA 1866 in wolves) (Fig. S16A, Supporting information) which is crucial for forming triglyceride-rich LDL particles (Young 1990) and is a conserved outer membrane channel domain (NCBI c121487). In *LIPG*, we identified a single missense mutation at position 420 causing an isoleucine (hydrophobic) to change to a threonine (polar). This mutation occurred within the PLAT domain of endothelial lipase (the protein encoded by *LIPG*) (Figs 6 and S16B, Supporting information). Previous functional protein assays have demonstrated that endothelial lipase has a unique 23 AA region in the PLAT domain that is likely to be crucial to the unique capabilities of endothelial lipase to interface with HDL particles (Razzaghi *et al.* 2013), and our mutation occurred near the beginning of that 23 AA region (Fig. 6). Finally, four highly ranked missense mutations occur within the longer isoform of *USH2A* (Fig. S16C, Supporting information). Two of these mutations, Ala2692Val and Asp2828Asn, were located within a region of *USH2A* consisting of nine fibronectin type III domains (NCBI cd00063). We also identified a three-base pair in-frame deletion (Ser1040del), predicted to be damaging by PROVEAN (Choi *et al.* 2012), within the functional laminin-type EGF-like motif domain (data not shown).

Discussion

Utility of study design

To test the ability of linked divergent SNPs to tag potentially functional variation, we assessed nucleotide variation in 980 genes. This effort provided an exhaustive assessment of the potential of genomewide SNP studies to study local adaptation. We then focused on a subset of genetic changes consistent with observed ecological differences among populations. We found support for the SNP genotyping approach, with up to 47% of the candidate genes identified with the SNP array genome scan (Schweizer *et al.* 2015) confirmed by resequencing as outliers with mutations that could affect function (nonsynonymous, deleterious nonsynonymous,

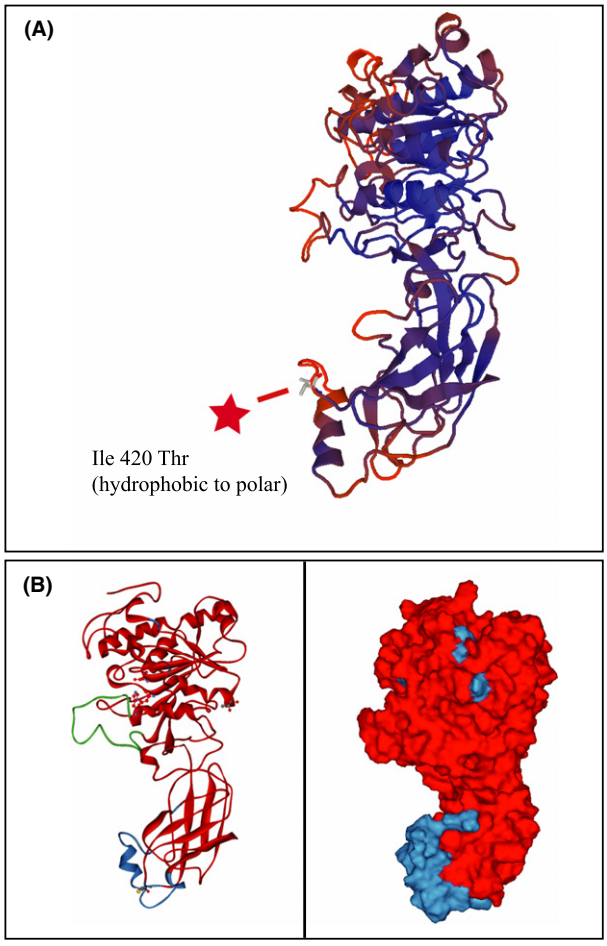

Fig. 6 Location of missense mutation within 3D protein structure of *LIPG*. (A) The mutation, which changes an Isoleucine (hydrophobic) to a threonine (polar) at amino acid (AA) position 420, occurs in a (B) 23 AA structural motif (blue helix structure) within the PLAT domain of *LIPG*. The full 3D structure of *LIPG* is provided for reference. Previous work indicates this domain may enhance the lipid binding function of LIPG. Part (B) is reproduced, with permission, from Razzaghi *et al.* (2013).

5′UTR, 3′UTR or TFBS mutations). Given that many of these regions were identified as outliers in the previous scan, their overlap with resequencing outliers is not necessarily an independent confirmation (Thornton & Jensen 2007). Nonetheless, resequencing reduced ascertainment bias in the genotype data as all variants, common and rare, were identified, thus enabling a higher-resolution examination of diversity. Wolves and dogs share ~99% of polymorphisms; however, SNPs on the genotyping array were chosen to be common in a panel of dogs, which may provide a biased view of genetic differentiation in wolves (vonHoldt *et al.* 2010; Schweizer *et al.* 2015). Our confirmation by resequencing suggests that the SNPs on the canine array are useful, despite ascertainment bias, for tagging linked outlier genes that have potentially functional variants (Haasl & Payseur 2015). We were able to computationally predict the effect of mutations through the use of SIFT and Miyata scores, and through detailed literature searches to identify functional domains within protein structures. Furthermore, the fact that many of our top candidates have been well studied in selection studies in multiple species supports the use of a priori candidate genes from other studies. In fact, 19 of 60 (32%) a priori candidates contained variants that were significant in at least one of our selection methods. Although we did find many more coding than noncoding variants in our selection candidates (Tables 1 and 2), we may have missed functional noncoding sequences due to limited sampling of flanking regions or inaccuracies in the TFBS databases. To further support our functional hypotheses based on coding or regulatory variation, future studies might utilize classical knock-in or knockout experiments in mice (Lewandoski 2001), new methods such as CRISPR to target specific alleles (Cong *et al.* 2013), or proteomic approaches (Diz *et al.* 2012).

Our use of extensive nongenic data ('neutral regions' verified using the dog genome annotation, CanFam3.1) as demographic controls offers an empirical method to potentially reduce the false positive rate and mitigate ascertainment bias inherent to SNP genotyping arrays. Genome scans can suffer from high rates of false positives as multiple evolutionary forces can produce similar genetic patterns of variation (Nielsen *et al.* 2007). Inclusion of a demographic model in the analysis can potentially address this problem, but models have simplifying, sometimes unrealistic assumptions or are too difficult to infer with modelling (Lotterhos & Whitlock 2014). Our results suggest that the modelling approach embedded in BAYESCAN may be too conservative (Foll & Gaggiotti 2008) as only a few outlier regions were resolved and were largely not shared in common with our other two outlier approaches (Figs S14 and S15, Supporting information). Therefore, in species with complex demographic histories, such as wolves, empirically based neutral controls may be preferable over explicit models that make specific demographic assumptions (Nielsen *et al.* 2005; Coop *et al.* 2010).

Finally, several important caveats should be noted about our experimental design. First, wolves have levels of LD allowing a moderately dense SNP array to tag genes within 10 kb (the distance at which $r^2 = 0.2$ in outbred populations; see Gray *et al.* 2009). Second, the dog SNP array was enriched for genic regions, with over 60% of SNPs tagging genes within 10 kb (Schweizer *et al.* 2015), which likely increased the efficacy of finding genes under selection, especially in comparison to random sequencing methods such as RAD-seq (Baird

et al. 2008; Jones & Good 2015). The use of an exome or transcriptome capture array (e.g. Bi *et al.* 2012) is an alternative to our approach that would provide complete sequences for potentially all transcribed genes in a single experiment, but few of those genes are likely to be under positive directional selection. For example, our SNP genotyping array identified a few thousand candidate genes from a total of about 12 000 tagged genes (Schweizer *et al.* 2015). Focusing capture on a reduced subset of genes allowed for higher coverage of each gene (>100×) and efficient use of sequencing resources (as many as 25 individuals per lane). In fact, we predict that double that number of individuals could have been sequenced in each lane and still allowed for high coverage (>50×) and accurate genotyping. One limitation is the availability of genic SNP arrays for the study species, but technological improvements will likely reduce the cost of construction and application of such genotyping arrays in the near future.

Temperature-related variation in immune-related genes

We predicted that variants among wolf ecotypes in immunity genes would change as a function of temperatures due to differences in pathogen prevalence (Allen *et al.* 2002; Guernier *et al.* 2004; Dionne *et al.* 2007). We found missense variants within two MHC Class II genes, *DLA-DQA* and *DLA-DRB1*, that were significantly associated in frequency and heterozygosity with altitude, temperature and percentage tree cover (Fig. 5, Table 1). Two beta-defensins, *CBD102* and *CBD103*, were also in sweep regions within Atlantic Forest and West Forest wolves, and an intron variant perfectly linked with the deletion causing black coat colour was significantly associated with temperature and land cover variables. The deletion variant of *CBD103* had previously been highlighted in Yellowstone and Canadian wolves for its possible function in coat colour and immunity (Anderson *et al.* 2009), and we show that it is also found in Atlantic and West Forest populations from Denali National Park. Significant GO categories of 'defence response' in both BAYENV and SWEED supported a role of immune response in these wolf ecotypes as well (Figs 3 and S10, Supporting information).

MHC Class II genes encode cell surface immune receptors that respond to bacterial antigens in the extracellular environment, and variation within these genes is thought to improve the defence response to pathogens (reviewed in Bernatchez & Landry 2003). Temperature-related variation in immunity genes has been observed in salmon, where clinal variation reflects changing vector prevalence in streams (Dionne *et al.* 2007). Heterozygote advantage has been documented in direct response to zoonotics (Osborne *et al.* 2015) and is associated with pathogen resistance (Bernatchez & Landry 2003). Likewise, we observed a correlation between heterozygosity for SNPs within MHC *DLA-DQA* and temperature variables (Fig. 5). In a previous study of MHC haplotype diversity in North American grey wolves (Kennedy *et al.* 2007), wolves of the boreal forest had the highest haplotype diversity at *DLA-DRB1*, *DLA-DQA1* and *DLA-DBQ1*, and the authors hypothesized that this pattern may be due to habitat-based isolation or postglacial recolonization history. Our results suggest that this pattern may also reflect temperature-related pathogen prevalence as we find that the frequency of the derived allele increases from 0.25 in High Arctic wolves to 0.55 in Boreal Forest wolves, the latter also have the highest mean temperature of the warmest month, relative to other populations (Fig. 5A). We observe similar patterns of correlation with temperature for the *CBD103*-linked intron variant (Fig. 4), with derived allele frequency increasing from 0.17 in High Arctic wolves to 0.57 in Boreal Forest wolves. Selective sweep regions containing immunity genes have also been identified in diverse species such as humans (Fagny *et al.* 2014), cattle (Qanbari *et al.* 2014), bank voles (White *et al.* 2013) and dogs (Akey *et al.* 2010).

Positive selection on vision, hearing and olfaction genes

We identified multiple candidate genes and GO categories related to vision, hearing and olfaction in wolves. For many of these genes, we found putatively selected missense mutations that have been implicated in human vision and hearing disorders (i.e. *PCDH15* and *USH2A* in Usher syndrome) or have been well studied in multiple organisms (olfactory receptor genes). Based on habitat-related variation in light and vegetation, and given that wolves are predators living in closed and open habitats, it is not surprising that divergent natural selection for vision, olfaction and hearing has occurred. Damaging mutations (SIFT score < 0.05) within *PCDH15* and *USH2A* were outliers for multiple environmental variables, and *PCDH15* was within a selective sweep region for Boreal Forest and Arctic wolves (Figs S5 and S6, Supporting information). For *PCDH15*, we observed an increase in derived allele frequency from near absence in Atlantic Forest wolves (0.06) to fixation (1) in High Arctic wolves, the latter of which experience the sharpest seasonal variation in light conditions. In humans, nonsynonymous mutations in *USH2A* are implicated in nonserious forms of deafness and oculta-neous albinism (Dreyer *et al.* 2000), while for *PCDH15*, large-scale genomic aberrations are more likely to cause

similar symptoms of Usher Syndrome (Le Guédard *et al.* 2007). *PCDH15* has also been identified as a candidate gene for selection related to echolocation in mammals (Parker *et al.* 2013), and as a gene within selective sweep regions in East Asian humans (Williamson *et al.* 2007; Grossman *et al.* 2010). Multiple sensory-related GO categories were also enriched in BAYENV (Fig. 3). Similarly, we predicted that differential ability to detect odorant molecules might be advantageous as a result of differing hunting conditions or intraspecific recognition factors across environments. We found multiple, damaging mutations within olfactory receptor genes, with allele frequency differences as much as 0.56 between Atlantic Forest and High Arctic wolves (Fig. 4). Together these data imply local adaptation at the molecular level in different wolf ecotypes mediated by environmental factors.

Olfactory receptor (OR) genes aid in sensing and distinguishing odorants in the environment and conspecifics from each other (reviewed in Ache & Young 2005) and are the most abundant gene class in canines with ~1100 genes (Quignon *et al.* 2005). Likely because of their functional importance, OR genes have been implicated in selection in multiple organisms, including primates (Gilad *et al.* 2003), canids (Chen *et al.* 2012b) and cattle (Qanbari *et al.* 2014). In naturally occurring populations of *Drosophila*, OR genes show clinal variation and signals of selection (Reinhardt *et al.* 2014). In our previous selection scan (Schweizer *et al.* 2015), we detected significant outliers in BAYENV that tagged OR genes. None of those genes had functional variants once resequenced here, which suggests that regulatory variants not captured here may have driven the signals on the SNP array. However, in this study we also found strong patterns in a different set of OR genes. Given that each OR gene detects a distinct odorant, and specific variants within OR genes have been demonstrated to affect odour perception (Keller *et al.* 2007; Keller & Vosshall 2008), our results suggest that different OR genes may be selected in wolf ecotypes in response to varying habitats.

Metabolism

We found striking examples of selection on metabolic genes in wolf ecotypes. Extreme environmental differences between the most distinct ecotypes (British Columbia, Arctic, High Arctic) and associated diet differences are hypothesized to select for genetic variants influencing lipid levels and insulin regulation for cold tolerance and varying levels of dietary fat (Schweizer *et al.* 2015). For *LIPG*, the gene encoding endothelial lipase, we found a missense variant that significantly correlated with both mean diurnal temperature range and precipitation seasonality, with the derived allele frequency rising from 0 in British Columbia wolves to 0.83 in High Arctic wolves (Fig. 4). This variant changes the amino acid from hydrophobic to polar within the functional PLAT domain (Fig. 6; Razzaghi *et al.* 2013). Likewise, in *APOB*, we found three significant missense mutations within the highly functional 26th exon that may affect the formation of triglyceride-rich VLDL particles. Interestingly, we also found that Arctic and High Arctic ecotypes had a large proportion of their GO-related categories (i.e. GO, KEGG, Reactome, human phenotype, microRNAs) represented by microRNA categories. MicroRNAs (miRNAs) are short segments of RNA that are involved in posttranscriptional regulation in many organisms and are increasingly implicated in adipocyte differentiation in humans and mice, and in response to environmental stress (Griffith-Jones 2004; Zaragosi *et al.* 2011; Hilton *et al.* 2012; Lyons *et al.* 2013; Wu *et al.* 2013; Storey 2015).

To our knowledge *APOB* and *LIPG* have not previously been identified as selection candidates in wolves, other than in our initial SNP array-based selection scan (Schweizer *et al.* 2015). Even so, both genes have been implicated in multiple diseases affecting humans and have been candidates under positive selection in other organisms. For instance, in a genomewide selection scan of polar bears and brown bears, *APOB* was one of the most statistically significant candidate genes, and contained mutations that may be functionally important for the high lipid diet of polar bears (Liu *et al.* 2014). Trout subjected to different fat content diets show differing expression levels of *LIPG* (Kolditz *et al.* 2008), and in humans, mutations in *LIPG* cause elevated HDL cholesterol (Edmondson *et al.* 2009; Razzaghi *et al.* 2013). *LIPG* and *APOB* are critical in the metabolism of HDL and LDL lipids, respectively, and are necessary for normal maintenance of lipid levels in the blood.

Pigmentation variation

We anticipated finding variants of genes involved in pigmentation pathways that may correspond to coat colour variation in wolves, and that function in camouflage (Jolicoeur 1959) or have secondary effects on immunity and fitness (e.g. Anderson *et al.* 2009; Coulson *et al.* 2011). We identified a selective sweep region within Boreal Forest and High Arctic wolves that included *CBD103*, and an intron variant within CBD103 in absolute linkage to the deletion haplotype that significantly varied with environmental variables. The frequency of the linked variant increases with increasing maximum temperature (Fig. 4), and also with documented coat colour frequencies (Gipson *et al.* 2002; Musiani *et al.* 2007; Anderson *et al.* 2009). Considering

this observed variation in wolf coat colour, it is intriguing that we found a missense mutation within *TYR* that is a significant outlier for percentage tree cover in BAYENV and located in a sweep region within Boreal forest and Arctic wolves. The same mutation was not significant in a genotype–phenotype association for white coat colour and was not found exclusively in white individuals (Fig. S17, Supporting information), suggesting it is one of several loci influencing colour variation (Barsh 1996; Hoekstra 2006; Sturm & Duffy 2012).

Lack of strong evidence for positive selection on morphological genes

Our evidence for selection on morphological variation was not as decisive as for other traits. We found this surprising given that size differences in wolves can facilitate more effective pursuit and capture of prey (MacNulty *et al.* 2009; Slater *et al.* 2009). We initially expected, given results from the SNP array-based genome scan (Schweizer *et al.* 2015), that we would find functional variants within genes having effects on skull morphology. However, we found no specific morphologically associated gene supported in the resequencing analysis. Conceivably, other genes or *cis*/*trans* factors that affect gene regulation and may influence morphological variation in wolves were not captured on the array. For example, we identified in our SNP array-based genome scan several genes within the BMP and WNT developmental pathways, but did not sequence them with our capture array due to design and space limitations.

Conservation implications

Our findings highlight local adaptation at the molecular level of wolf ecotypes in North America. Two of the ecotypes showing the greatest number of unique outlier genes are from the Arctic and High Arctic (Fig. 2). Unfortunately, these wolf ecotypes inhabit tundra environments that may disappear by the end of this century (Mech 2004; Gilg *et al.* 2012; Mahlstein & Knutti 2012), and are threatened by human impacts such as hunting (Musiani & Paquet 2004; Bryan *et al.* 2014). Most notably, we detect positive selection in Arctic and High Arctic wolves on genes influencing vision, immunity, pigmentation and metabolism (Fig. 4). The high level of adaptive distinction found in these ecotypes might be expected given the extreme environment in which they live, but our molecular results provide a powerful mandate to enhance protection of these populations as they represent the most adaptively distinct North American wolves that we have sampled. The large number of GO-related category types in Arctic wolves demonstrates highly specific adaptations to their environment (Fig. 2A). The large number of significantly enriched microRNA categories (>100) and the literature implicating microRNAs in adipocyte differentiation and extreme environment adaptation implies that Arctic wolves may have evolved regulatory responses to their environment (Fig. 2A). Similarly, we find that British Columbia coastal wolves have a unique suite of molecular adaptations that support arguments for adaptive distinction (Muñoz-Fuentes *et al.* 2009). Differing sample sizes are unlikely to drive these patterns, as High Arctic and Arctic represent sample sizes at either extreme, but have similar numbers of genes and GO-related categories. The use of the relative number of genes and the top-level GO-related categories under selection could potentially add to metrics for ranking conservation priorities based on the need for the preservation of adaptive diversity (Bonin *et al.* 2007; Gebremedhin *et al.* 2009). Specifically, the number of genes under selection provides a numerical ranking of adaptive diversity in each population akin to species diversity indices, whereas the GO categories represented by these genes are more similar to a higher-order taxonomic grouping, such as genus or family. Therefore, those populations having the greatest number of unique genes and GO categories could be argued to deserve the greatest priority for conservation of adaptive diversity. Although GO categories are related and hierarchical, these simple indices are a possible alternative to other schemes for prioritizing the management of adaptive diversity (Fraser & Bernatchez 2001; Funk *et al.* 2012) and represent genomewide measures of adaptive divergence that can readily be incorporated into conservation schemes.

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R.M.S., J.A.R., R.H., P.S., M.G., M.M., R.E.G., J.N. and R.K.W. designed research. R.M.S., J.A.R., R.H., P.S. and M.G. performed research. P.S., M.M. and R.E.G. contributed new reagents or analytical tools. R.M.S., R.H., R.E.G., J.N. and R.K.W. analysed the data. R.M.S. and R.K.W. wrote the manuscript.

Data accessibility

Sequence reads and mapping files are archived at the NCBI SRA under SRP065570. The variants file and sample information (locations, climate data, phenotype) have been uploaded with data analysis results files to Dryad (doi: 10.5061/dryad.8g0s3).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Supplemental methods for sample processing and candidate gene descriptions.

Fig. S1 Mean depth of coverage of neutral and genic capture regions for each wolf sampled on the capture array.

Fig. S2 Significantly enriched gene ontology (GO) categories containing genes with functional variants as annotated by Variant Effect Predictor, SIFT, and our TFBS databases (see methods for details).

Fig. S3 Significantly enriched human phenotype (hp) categories containing genes with functional variants as annotated by Variant Effect Predictor, SIFT, and our TFBS databases (see methods for details).

Fig. S4 Manhattan plot of the SWEED composite likelihood ratio (CLR) score for each genic position within West Forest wolves.

Fig. S5 Manhattan plot of the SWEED composite likelihood ratio (CLR) score for each genic position within Boreal Forest wolves.

Fig. S6 Manhattan plot of the SWEED composite likelihood ratio (CLR) score for each genic position within Arctic wolves.

Fig. S7 Manhattan plot of the SWEED composite likelihood ratio (CLR) score for each genic position within High Arctic wolves.

Fig. S8 Manhattan plot of the SWEED composite likelihood ratio (CLR) score for each genic position within British Columbia wolves.

Fig. S9 Manhattan plot of the SWEED composite likelihood ratio (CLR) score for each genic position within Atlantic Forest wolves.

Fig. S10 Significantly enriched gene ontology (GO) categories containing genes with significant ($P \leq 0.01$) outliers from SWEED.

Fig. S11 Significantly enriched human phenotype (hp) categories containing genes with significant ($P \leq 0.01$) outliers from SWEED.

Fig. S12 Unfolded site frequency spectra (SFS) for neutral, genic, 0-fold degenerate, 4-fold degenerate, and selective sweep sites in (A) West Forest, (B) Boreal Forest, (C) Arctic, (D) High Arctic, (E) British Columbia, and (F) Atlantic Forest ecotypes.

Fig. S13 Significantly enriched human phenotype (hp) categories containing genes with significant ($P < 0.005$) outliers from BAYENV.

Fig. S14 Venn diagram showing overlap between candidate genes from SWEED, BAYESCAN, and BAYENV at two significance thresholds (top, red is $P \leq 0.01$; bottom, blue is $P \leq 0.05$).

Fig. S15 Venn diagram showing overlap between candidate genes from SNP array and capture array for each selection test.

Fig. S16 Protein alignments between human and dog for (A) APOB, (B) LIPG, and (C) USH2A.

Fig. S17 Correlation of allele frequency of missense TYR with wolf coat color.

Table S1 List of genes, gene names, and their references, for 60 a priori candidate genes.

Table S2 Summary statistics and number of positions within genic and neutral regions.

Table S3 Genotype concordance between Affymerix SNP array and resequencing data.

Table S4 Pearson's correlation between maximum test statistic and gene length within SWEED and BAYENV.