Cold adaptation drives population genomic divergence in the ecological specialist, *Drosophila montana*

Wiberg, R. A. W.[1,+], Tyukmaeva, V.[2+], Hoikkala, A.[2], Ritchie, M. G.[1] & Kankare, M.[2,*]

1. Centre for Biological Diversity, School of Biology, University of St Andrews, St Andrews, KY16 9TH, Scotland, UK. **2.** Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, 40014, Finland. **+Present addresses: RAWW** University of Basel, Department of Environmental Sciences, Zoological Institute, Vesalgasse 1, 4051 Basel, Switzerland **TV** Centre d'Ecologie Fonctionelle et Evolutive, CNRS, Montpellier, France.

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ABSTRACT

Detecting signatures of ecological adaptation in comparative genomics is challenging, but analysing population samples with characterised geographic distributions, such as clinal variation, can help identify genes showing covariation with important ecological variation. Here we analysed patterns of geographic variation in the cold-adapted species *Drosophila montana* across phenotypes, genotypes and environmental conditions and searched for signatures of cold adaptation in populations' genomic divergence. We first derived the climatic variables associated with the geographic distribution of 24 populations across two continents to trace the whole scale of environmental variation experienced by the species, and measure variation in the cold tolerance of the flies of six populations from different geographic contexts. We then performed pooled whole genome sequencing of these six populations, and used Bayesian methods to identify SNPs where genetic differentiation is associated with both climatic variables and the population phenotypic measurements. The top candidate SNPs were enriched on the X and 4th chromosomes, and they also lie near genes implicated in other studies of cold tolerance and population divergence in this species and its close relatives. We conclude that ecological adaptation has contributed to the divergence of *D. montana* populations throughout the genome and in particular on the X and 4th chromosomes, which also showed highest interpopulation F_{st}. This study demonstrates that ecological selection can drive genomic divergence at different scales, from candidate genes to chromosome-wide effects.

INTRODUCTION

The geographic structure of a species is a result of its phylogeographic history, influenced by past and present dispersal, population demography and selection. Obtaining genome-wide data on SNP genetic polymorphisms across multiple populations of a species is becoming relatively easy, but interpreting the patterns of geographic variation in such data and identifying genes which vary primarily due to selection remains challenging. Often a simple 'outlier' approach using genome scans which measures genetic differentiation such as F_{st} or Dxy is adopted, but results are difficult to interpret due to confounds between selection, drift and population structure, or genomic features such as inversions and other causes of variation in recombination rate (Noor & Bennet, 2009; Cruickshank & Hahn, 2014; Wolf & Ellegren, 2016; Ravinet et al., 2017). If environmental data is available, we can use associations with such factors to help identify loci where differentiation covaries with this environmental variation. Genome scan methods can incorporate environmental variation and simultaneously fit effects for covariance with environmental factors, and other population processes. This approach has successfully identified genetic variation associated with altitude in humans, among other examples (Foll, Gaggiotti, Daub, Vatsiou, &

Excoffier, 2014; de Villemereuil & Gagiotti, 2015; Gautier, 2015) and has become a useful approach to investigate the ecological adaptations underlying population divergence.

Clinal patterns of variation in phenotypes or gene frequencies have a long history of being used to infer selection along ecotones, and analyses of cline shape can sometimes identify loci under direct selection from others showing clinal variation for other reasons such as phylogeographic history (Barton & Gale, 1993). Such studies can be very powerful, especially when independent parallel clines are available. For example, Kolaczkowski, Kern, Holloway & Begun (2011) sampled isofemale lines from extremes of a cline in Australian populations of *D. melanogaster*, and found many genes implicated in clinaly varying phenotypes to show highest differentiation. Also, Bergland, Behrman, O'Brien, Schmidt & Petrov (2014); Kapun, Fabian, Goudet & Flatt (2016) sampled North American clines in *D. melanogaster* and *D. simulans* over several years and seasons to uncover clinal and seasonal variation in genome scale. They found that many SNPs showed consistent seasonal fluctuations in allele frequencies throughout the clines, which indicates a regular response to seasonally varying selection pressures (Bergland et al., 2014). On the other hand, Machado et al. (2015) concluded that migration and gene flow play a greater role than adaptation in the overall clinality of genomic variants in *D. simulans* than *D. melanogaster*. While the two species share a significant proportion of the genes showing clinal variation, their differences in overwintering ability, migration and population bottlenecks probably act as additional drivers of differences in patterns of variation between them (Machado et al., 2015). Similar studies of clinal variation in phenotypes and allele frequencies have also been carried out in other insects (e.g. Paolucci, Salis, Vermeulen, Beukeboom, & van de Zande, 2016), plants (e.g. Chen et al., 2012; Bradbury, Smithson, & Krauss, 2013), mammals (e.g. Hoekstra, Drumm, & Nachman, 2004; Carneiro et al., 2013), fish (e.g. Vines et al., 2016), and other organisms (Endler, 1973; Endler, 1977; Takahashi, 2015). However, the patterns of variation in allele frequencies are only rarely compared with potentially causal environmental variation or ecologically important traits, even though such studies are necessary to determine if adaptation to climate is directly driving patterns of genetic differentiation.

Here we investigate geographic variation at both the phenotypic and genetic level in *Drosophila montana* samples from two continents. This species has spread around the northern hemisphere (Throckmorton, 1969), and is one of the most cold-tolerant *Drosophila* species (Kellerman et al., 2012; Vigoder et al., 2016). The basic cold tolerance of *D. montana* flies can increase towards the cold seasons through two mechanisms, photoperiodic reproductive diapause (Vesala & Hoikkala, 2011) and cold-acclimation induced by a decrease in day length and/or temperature (Vesala, Salminen, Laiho, Hoikkala, & Kankare, 2012; Kauranen et al., 2019). *D. montana* populations have been found to show clinal variation in the critical day length required for

diapause induction (CDL) and its temperature-sensitivity. There is also a correlation between CDL and latitudinally co-varying climatic factors such as the mean temperature of the coldest month (Tyukmaeva, Lankinen, Kinnunen, Kauranen, & Hoikkala, 2020). In addition, *D. montana* populations from different geographic regions show variation in their courtship cues and mate choice (Routtu et al., 2007; Klappert, Mazzi, Hoikkala, & Ritchie, 2007), which has led to partial reproductive isolation between some distant populations (Jennings, Mazzi, Ritchie, & Hoikkala, 2011; Jennings, Snook, & Hoikkala, 2014). At the genetic level, differential gene expression studies have identified candidate genes underlying diapause (Kankare, Salminen, Laiho, Vesala, & Hoikkala, 2010; Kankare, Parker, Merisalo, Salminen, & Hoikkala, 2016), perception of day length (Parker, Ritchie, & Kankare, 2016), and cold acclimation (Parker et al., 2015). Furthermore, a quasi-natural selection experiment for shorter CDL, accompanied by a decrease in cold-tolerance, induced widespread changes in loci with potential roles with these traits (Kauranen et al., 2019). Finally, population genomic analyses has identified several outlier loci when examining differentiation between North American and European populations (Parker et al., 2018). All this makes *D. montana* an interesting example of nascent speciation, potentially influenced by ecological adaptation.

Here we ask to what extent the patterns in the genomic divergence of populations across continents are correlated with climatic variation and phenotypic responses to cold adaptation. We perform pooled whole genome sequencing (pool-seq) on six different populations and use Bayesian methods to examine the association between genomic differentiation between populations and environmental variables across both continents. We also phenotyped populations for two different cold tolerance measures, critical thermal minimum (CTmin) and chill coma recovery time (CCRT), and investigate the associations between them and the genetic and climatic data. Ultimately, we ask if the genomic loci showing an association between genetic and environmental differentiation also show association with population differentiation in cold tolerance phenotypes, and examine the possible overlap between the set of genes close to candidate SNPs with sets of candidate genes from other studies of cold adaptation in *D. montana*. If population differentiation is driven by ecological selection then we would predict the extreme cold adaptation of *D montana* to have left a signature of genomic divergence across these loci.

METHODS

Sample collections and DNA extractions

We collected samples of 49-50 individuals from six *D. montana* populations from a range of latitudes from 66°N to 38°N in the spring of 2013 or 2014. Four of these populations represented a latitudinal cline in North America (N.A.), and two populations were from a latitudinal cline in

Finland (figure 1; table 1). Samples of wild-caught flies from the six populations were stored in Ethanol (the male/female ratio varied across samples; table 1) and DNA of individual flies was extracted using CTAB solution and phenol-chloroform-isoamylalcohol purifications in 2016. Genomic DNA was extracted from individual flies and quantified using Qubit (Thermo Fisher Scientific), and an equal amount of DNA from each individual (50 ng) was pooled into the final sample. Sequencing was performed at the Finnish Functional Genomics Centre in Turku, Finland (www.btk.fi/functional-genomics) on the Illumina HiSeq3000 platform (paired-end reads, read length = 150bp, estimated coverage ~121x).

Phenotyping

We measured the critical thermal minimum (CTmin) and chill coma recovery time (CCRT) of flies from six populations. Fly samples for these tests were collected for five populations (Seward, Terrace, Ashford, Crested Butte and Korpilahti) from population cages that have been maintained in the lab since 2013-2014. For the Oulanka population flies were collected from three isofemale strains (established in 2014), because the population of this population had been contaminated by another species. Newly emerged flies (<24 hours old) were anesthetized with CO_2, separated by sex and kept in vials with yeast-malt media (Lakovaara, 1969) at constant light at 19°C. The vials were changed weekly, until the flies reached sexual maturity at the age of 21-22 days and were used in CTmin and CCRT tests. The same individual flies were first used in the CTmin experiment, then followed by the CCRT test.

CTmin and CCRT tests were done in batches (21 in total) for 39 flies per population per sex on average (for Oulanka isofemale strains 30 flies per strain per sex were used). CTmin tests are based on detecting the temperature (CTmin) at which flies lose neuromuscular function and enter reversible state of chill coma (Andersen et al., 2015). In these tests, the flies were placed into tubes sealed with parafilm and submerged into a 30% glycol-water mixture in Julabo F32-HL chamber. The temperature was decreased at the rate of 0,5°C per minute (in range from 19°C to -6°C) and CTmin was determined as the temperature at which a fly was unable to stand on its legs. After determining the flies' CTmin, the temperature was increased to -6°C and the flies were left in this temperature for 16 hours. Then vials were quickly taken out of the glycol-water bath and the flies' CCRT was determined as the time required for the flies to recover from chill coma and stand on their legs.

To investigate population differences in CTmin and CCRT phenotypes we fit linear mixed models (in R v. 3.6.2; R Development Core Team, 2019) using the "lme4" R package; Bates, Mächler, Bolker, & Walker, 2015). The full model included population and sex as fixed effects and experimental batch as a random effect. Fixed effects were tested by sequential comparison by a

likelihood ratio test and removed if they did not significantly improve the model fit (see supplementary R scripts).

Bioclimatic variables and population geography

We obtained representative climate data from the WorldClim database (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) for each *D. montana* population sampled for the pool-seq (see above), as well as for 18 additional populations of this species used in our other studies, the R package "raster" (v. 2.5-8; Hijmans et al., 2016) in R. In total this amounts to 55 bioclimatic variables for each population (table S1). To reduce the number of variables in the dataset a principle components analysis (PCA) was performed using the "PCA()" function from the "FactoMineR" package (v. 1.28; Lê, Josse, & Husson, 2008) in R. Principle components were kept for further analysis if their eigenvalues were > 1. PCA scores for each population were z-transformed using the "scale()" function in base R. Additionally, CTmin and CCRT were summarised to a mean value for each population. In total, this gives four "environmental" variables measured for each population (PC1, PC2, CTmin, and CCRT).

Mapping, SNP calling and genomic analysis

Quality of reads was checked with FASTQC (v. 0.11.5) (Andrews, 2015) and reads were trimmed using trimmomatic (v. 0.32) (Bolger, Lohse, & Usadel, 2014). Trimmed reads were mapped to the *D. montana* reference genome (Parker et al., 2018) using BWA mem (v. 0.7.7) (Li, 2013) with the default options but keeping only alignments with a mapping quality of > 20 following best practice guidelines for pool-seq (Schlötterer, Tobler, Kofler, & Nolte, 2014). Duplicate alignments were removed with samtools rmdup (v 1.3.1) (Li et al., 2009) and regions around indels were re-aligned using picard (v. 1.118, Broad Institute), GATK (v. 3.2-2, McKenna et al., 2010) and samtools. Separate .bam files for each of the sequenced sample were finally merged using bamtools (v. 2.4.0; Barnett, Garrison, Quinlan, Strömberg, & Marth, 2011).

Over 80% of reads were properly mapped in all samples. Empirical coverage was between ~100 and 110x (table S2; figure S1) while the mean coverage for Seward samples was nearly twice that of the other samples (figure S1 and figure S2). To avoid the potential for this difference causing artefacts in downstream analyses the .bam files for Seward were down-sampled to contain 94.1 million reads (the average across the remaining populations). The coverage was then much more similar among the populations, allowing common maximum and minimum thresholds to be set based on the aggregate distribution (figure S3). Allele frequencies at SNPs among the pools were called with samtools mpileup (v. 1.3.1; Li et al., 2009) using options to skip indel calling as well as ignoring reads with a mapping quality < 20 and sites with a base quality < 15. This was followed by

the heuristic SNP calling software PoolSNP using a minimum count of five to call an allele, and a minimum coverage of 37 and a maximum coverage < 95th percentile of the scaffold-wide coverage
192 distribution to call a SNP (Kapun et al., 2019).

To test for an association between the four environmental variables and genetic differentiation we used BayeScEnv (v. 1.1; Foll & Gaggiotti, 2008; de Villemereuil & Gaggiotti,
195 2015). BayScEnv was run with five pilot runs of 1000 iterations each in length followed by a main chain of 4,000 iterations of which 2,000 were discarded as burn-in. Four MCMC chains were run for each analysis to evaluate convergence of parameter estimates. Because of the unbalanced
198 number of males (and therefore ratios of X:Y chromosomes) in the pools, BayScEnv analyses were performed separately on SNPs that could be assigned to the autosomal linkage groups (chromosomes) and the X chromosome. Raw count data were used for the autosomal data. For X
201 linked SNPs, allele count data were scaled to the known number of X chromosomes in the pool using n_{eff}, the effective sample size taking into account the multiple rounds of binomial sampling inherent to a pool-seq design (Kolaczkowski et al., 2011; Feder, Petrov, & Bergland, 2012).

204 Chains were assessed for convergence with the "coda" R package (v. 0.19-1; Plummer, Best, Cowles, & Vines, 2006). Convergence was good across the four chains for most analyses and parameters (potential scale reduction factors (PSRFs) of ~1 in a Gelman-Rubin diagnostic test;
207 figures S4-S7), except for analyses of autosomal SNPs and PC2 as the environmental variable which showed mild convergence problems (PSRF = 1.71), although parameter estimates agreed well with the other chains. Thus, this first chain was discounted for all analyses and only estimates
210 from the remaining 3 chains were used. The union of significant SNPs (q-value for the g parameter < 0.05) across these chains were taken as the final candidate SNPs.

Finally, population genetic statistics (π, and Tajima's D) were computed in windows of 10kb
213 with a step size of 5kb using methods implemented for pool-seq data (Kapun et al., 2019). These statistics were only computed for scaffolds with a length > 10kb. F_{st} was computed for each population with the R package "poolfstat" (v. 1.1.1; Hivert, Leblois, Petit, Gautier, & Vitalis, 2018)
216 by first computing all pairwise values, and then deriving population specific F_{st} values by averaging across all pairwise values where a population was included. See the supplementary material for pseudocode commands of the key pipeline steps.

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RESULTS

Cold tolerance measures, bioclimatic variables and population geography

222 Across individuals, there was no evidence of an association between minimum critical temperature (CTmin) and the chill coma recovery time (CCRT; cor = 0.08, p = 0.07). CTmin and CCRT varied among populations according to sex, population and/or latitude. CTmin was

significantly different between sexes (X^2 = 10.1, d.f. = 1, p = 0.002) as well as among populations (X^2 = 51.4 , d.f. = 5, p < 0.001) but there was no evidence for an interaction (X^2 = 4.8, d.f. = 5, p = 0.44; see also figure 2A). Similarly, CTmin covaried significantly with latitude (X^2 = 7.9, d.f. = 1, p = 0.005) and sex (X^2 = 11.3, d.f. = 1, p < 0.001), but there was no significant interaction effect between latitude and sex (X^2 = 0.47, d.f. = 1, p = 0.49). For CCRT there was no effect of sex (X^2 = 1.6, d.f. = 1, p = 0.2) but it varied significantly among populations (X^2 = 18.5, d.f. = 5, p = 0.002; figure 2C) and, despite the striking difference between males and females in the Seward population (figure 2C), there was no evidence for a significant interaction effect between population and sex (X^2 = 4.38, d.f. = 5, p = 0.5). Similarly, only latitude had a significant effect on CCRT (X^2 = 16.9, d.f. = 1, p < 0.001). As one would expect, CTmin showed on average lower values (figure 2B), and CCRT times were shorter (figure 2D), at higher latitudes, meaning that more northern populations show higher cold tolerance. The final models for CTmin and CCRT are given in the supplementary material.

We performed Principal Component Analysis (PCA) of the WorldClim climate data for a total of 24 *D. montana* populations, from which we had collected samples and where climate data were available. This enabled us to ensure that the populations, which we had chosen for the cold tolerance tests and genome sequencing, represented the whole scale of environmental variation experienced by the species. These analyses identified four Principal Components (PCs) that together explained about 98% of the variation (figure 3A and B). The first two PCs separated the populations roughly by a measure of "distance inland" (PC1) and then by latitude (or altitude) (PC2). PC1 explained ~55% of the variation (figure 3C and D) and loaded heavily on climate and biological variables associated with precipitation and temperature such as "Mean Temperature of Coldest Quarter", "Precipitation of Wettest Month", "Annual Precipitation". Meanwhile, PC2 explained about 23% of the variation and loaded heavily on biological variables that are associated with latitudinal clinality, e.g. "Mean Diurnal [Temperature] Range," and "Isothermality" which is the diurnal range divided by the mean "Annual [Temperature] Range". The remaining PCs (PC3 and PC4) explained about 11.5 and 5% of the variation respectively and did not capture as much of the climatic variation. Neither latitude (Spearman's Rank Correlation: rho = -0.50, p = 0.02) nor altitude (rho = -0.37, p = 0.12) correlated significantly with PC1. However, both altitude (rho = -0.56, p = 0.01) and latitude (rho = -0.59, p = 0.003) correlated with PC2 (see also figure 3). Importantly, all these patterns also hold if PCA is performed using only the 6 populations for which genomic data were collected. Thus, any relationship between environmental variables and genetic differentiation in the samples selected for pool-seq is likely to reflect true patterns across populations of *D. montana*. To examine the association between climate and phenotype, we compared these across the six populations. CTmin was positively correlated with PC1 (correlation

coefficient (cor) = 0.94, $p < 0.01$) and with PC2 (cor = 0.75, $p = 0.09$). However, CCRT showed no relationship with either PC1 (cor = 0.1, $p = 0.85$) or PC2 (cor = -0.45, $p = 0.37$) although the small sample sizes (N = 6 in all cases) makes reliable conclusions difficult.

Genomics

The number of SNPs with a significant association between overall F_{st}, the two cold tolerance measures, and the two PCs of the bioclimatic data varied from 612 and 2,480 (table 2). Using PC1 as an environmental variable with BayeScEnv gave a total of 2,976 and 1,528 SNPs with a q-value < 0.05 on the autosomes and on the X chromosome respectively. Interestingly, the distribution across the chromosomes was not random as, by using the distribution of all SNPs as the expected distribution, there was a significant deviation from expectation ($X^2 = 2{,}906.4$, d.f. = 4, $p < 0.001$). There were many more SNPs than expected on chromosome 4 (1,432 vs. 954) and on the X chromosome (1528 vs. 526). Results were similar for PC2 with 6,607 and 1,861 SNPs with a q-value < 0.05 on the autosomes and X chromosomes, respectively. Again, there was a significant deviation from the expected distribution of SNPs across the chromosomes ($X^2 = 1{,}681.9$, d.f. = 4, $p < 0.001$) with an overrepresentation on the 4th (2,480 vs. 1,794) and the X chromosomes (1,861 vs. 989).

We also used average CTmin values per population in similar analyses and found a total of 2,668 and 1,272 SNPs with a q-value < 0.05 on the autosomes and X chromosomes, respectively. The pattern of significant deviations from expected distributions ($X^2 = 2{,}526.2$, d.f. = 4, $p < 0.001$) was also due to an excess on the 4th (1,383/835) and the X chromosomes (1,272/460). Similar results were found for CCRT with a total of 2,240 and 1,228 SNPs with a q-value < 0.05, respectively. Once again, there was a significant deviation from the expected distribution of SNPs ($X^2 = 2{,}825.6$, d.f. = 3, $p < 0.001$) with and excess on the 4th (1,252/735) and the X chromosomes (1,228/405).

To examine the loci implicated in the four BayScEnv analyses, we identified genes containing or nearby (within 10kb) the candidate SNPs (table S3). The second largest set are those genes unique to the analysis of PC2 as an environmental covariate (figure 4). However, overall there is substantial overlap among these genes with ~39% (1,102 in total) of them being shared by all the four analyses (table S3, figure 4). Some (147, ~13%) of these common genes are novel to *D. montana* (i.e. not annotated in *D. virilis* or other *Drosophila* spp.) and therefore have no annotation, but 955 have an identifiable *D. virilis* ortholog (table S3). Functional enrichment analyses revealed several common categories of genes associated with the climatic variables and population phenotypes (table 3, all categories are given in table S4). For example, terms associated with membrane and transmembrane structures, immunoglobulins, HAD hydrolase and nucleotide

binding were enriched in most of the variables (table 3). Interestingly, there were also several gene ontology categories that were only enriched in one of the variables, like glycoside and ATPase hydrolase in CCRT and ion channels and transport, as well as metal binding in PC1 (table 3, table S4).

We then compared these loci with genes implicated in previous studies of climatic adaptation in *D. montana* including gene expression studies of traits connected to diapause and cold-tolerance (Kankare et al., 2010; 2016; Parker et al., 2015; 2016). Additionally, several outlier genes have been identified near the most significantly differentiated SNPs among *D. montana* populations from Oulanka (Finland), and from North American populations in Colorado and Vancouver (Parker et al., 2018). Finally, experimental selection experiments identified several genes near SNPs responding to selection from changes to the photoperiod (Kauranen et al., 2019). We tested for an overlap between the total set of genes within 10kb of outlier SNPs from all of the BayScEnv runs (N = 2,694) and the candidate gene sets identified in earlier studies (see table S5 for the gene sets and studies used). We computed a bootstrap distribution of overlaps by sampling 2,694 random genes from the *D. montana* annotation. For each gene set this was done 100 times and the distribution compared to the empirical overlap. Results are given in figure S8. In all the cases the empirical overlap was greater than expected by chance. The only gene that was found in all five of the previous studies used and in the comparison here is called *sidestep II* (*side-II*; table S6). Unfortunately, there is no information available about the biological processes or molecular functions connected to it. Moreover, from 44 other genes that were common to four of our previous studies and this study (table S6) most (27) have an ortholog in *D. melanogaster*. These genes have molecular functions such as transmembrane signaling or transporter, acetylcholinesterase, ATP binding, protein serine/threonine kinase, carboxylic ester hydrolase, or Rho guanyl-nucleotide exchange factor activity (Thurmond et al., 2019). Many of the genes are also connected to metal ion, nucleid acid or zinc ion binding (table S6). After identifying information on molecular or biological function and Interpro domains, eventually only five genes remained for which there was no information available (table S6).

Finally, examination of population genetic parameters identified the Crested Butte population as anomalous. The distribution of Tajima's D is centered close to zero in most populations, being slightly more negative in North American populations (figure S9). However, Crested Butte is an outlier with a greatly reduced genome-wide Tajima's D (figure S9). Similarly, diversity (π) is also lower in this population than in other populations. There is no overall relationship between latitude and π (Spearman's rho = 0.14, N = 6, p = 0.8; figure S9) but there is a strong correlation between latitude and Tajima's D which is influenced by this population (with Crested Butte: rho = 0.88, N = 6, p = 0.03; without Crested Butte: rho = 0.8, N = 5, p = 0.13).

330 Although Crested Butte occurs at a much higher altitude (>2800 meters) than other populations neither Tajima's D nor π correlated significantly with altitude (Tajima's D: rho = -0.6, N = 6, p = 0.24, π: rho = -0.6, N = 6, p = 0.24). Furthermore, F_{st} was similar across all populations and 333 chromosomes with the exception of Crested Butte which remained an outlier with unusually high F_{st} (table 4). Finally, F_{st} was always highest on chromosome 4 and the X chromosome, complementing the results seen in BayeScEnv analyses (table 4 *c.f.* table 2) and as expected if this ecological 336 selection is influencing genomic divergence.

DISCUSSION

339 Detecting genomic signatures of climatic adaptation is an important, but challenging task. Here we use multiple sources of evidence to study ecological adaptation and population divergence in a highly cold tolerant species of *Drosophila*, *D. montana*. This species is characterised by a wide 342 circumpolar distribution extending to high latitudes both in North America and Europe, and to high altitudes in the southern part of its range in the Rocky Mountains of North America. These habitats impose extreme seasonal and climatic selective pressure. In this study, we collected bio-climatic 345 data from 24 populations along a latitudinal gradient of about 2,900 km in North America, and six populations from a gradient of 720 km in Finland. We then characterised population level cold-tolerance for six populations from these clines using two commonly used cold tolerance methods, 348 critical thermal minimum (CTmin) and chill coma recovery time (CCRT). We also performed pool-seq of six of these populations to investigate the association between genomic and environmental differentiation across different populations.

351 In our study, two methods examining cold tolerance gave somewhat different results, as CTmin, but not CCRT, differed significantly between sexes, with females having lower CTmin values than males, on average. Similarly, in an earlier study investigating seasonal changes in *D.* 354 *montana* CCRTs, only one out of six comparisons showed a significant difference between sexes (Vesala, Salminen, Kostal, Zahradnickova, & Hoikkala, 2012) and Gibert, Moreteau, Petavy, Karan & David (2001) did not detect sex-specific differences in CCRT in any of 84 *Drosophila* species 357 they studied. However, several studies of *D. melanogaster* have detected shorter CCRT in females than in males, suggesting that females are more cold tolerant than males (David et al., 1998; Andersen et al., 2015; Bauerfeind, Kellermann, Moghadam, Loeschcke, & Fischer, 2014) which 360 could be related to their greater body mass (e.g. Wilder et al., 2010). Consequently, the extent and adaptive significance of sex-specific differences in CCRT in *Drosophila* remains unclear. Overall, regardless of the sex, CTmin were lower and CCRT times shorter in higher latitude populations as 363 one would expect, indicating that more northern populations are more cold-tolerant.

We then derived principal components to summarise WorldClim climatic variables using data from all the 24 populations of *D. montana* spanning two continents. The first principal component (PC1) separated populations roughly by a measure of "distance inland" and loaded heavily on climate and variables associated with precipitation and temperature. These results follow the geographic distribution of the populations, for example, the population with highest values for PC1 is Ashford, which is a population on the Pacific coast and receives most rain, but also experiences warm summers and mild winters. Meanwhile, principal component 2 (PC2), loaded heavily on bioclimatic variables associated with latitudinal clinality, which also mapped onto location of the populations intuitively as populations with higher values on PC2 also occurred at higher latitudes. Interestingly, CTmin values were positively correlated with PC1, but not with PC2, while CCRT showed no relationship with either of these components. This suggests that CTmin and CCRT measure at least slightly different biochemical or physiological mechanisms as e.g. MacMillan, Williams, Staples, & Sinclair, 2012 and Findsen, Pedersen, Petersen, Nielsen & Overgaard (2013) have suggested, and could hence be correlated with different climatic variables. Indeed across the individuals measured here these two traits are also uncorrelated, and the correlation between these two measures has often been found to be relatively weak in other studies (Andersen et al., 2015).

The Bayesian analysis identified SNPs showing an association of genetic differentiation with climatic and phenotypic variation. The extent to which the loci underlying the phenotypes and adaptations to different climatic conditions are shared indicates that these are closely associated in driving genome evolution. We found that genes near SNPs showing a significant association between genetic and climatic differentiation overlapped to a large extent with genes near SNPs showing a significant association between genetic and phenotypic differentiation among populations. The largest intersection set, containing 1,102 genes, was the one containing genes near SNPs associated with all the four variables examined (PC1, PC2, CTmin and CCRT). However, PC2 loads heavily on bioclimatic variables relating to latitude, and our analysis using PC2 as a covariate has a large number of private genes (see figure 4), suggesting that there is also a substantial amount of genetic variation underlying adaptations to latitude unrelated to the phenotypic measures we have quantified. Our study is an excellent example of how strong ecological selection may be detected in genomic studies. In particular, because Bayesian methods examining both ecological variables and relevant phenotypes gives significant overlap amongst the associated loci, and that these are further associated with more broad genomic differentiation between populations, gives confidence that we are consistently identifying genes associated with ecological selection.

Analyses of the functional annotation of these genes strengthens our conclusions that climate driven adaptation is important. Regions near SNPs significantly associated with climatic variables were enriched for genes previously identified as candidates related to cold tolerance, diapause and responses to changes in day length in *D. montana* (Kankare et al., 2010; 2016; Parker et al., 2015; 2016). Thus, genetic variation across populations of these flies may be largely shaped by differences in ecological and climatic variation. This ecological specialisation may have also contributed to the divergence of *D. montana* from its relatives. Parker et al., (2018) surveyed the rates of molecular evolution in eleven cold tolerant and non-cold tolerant species of Drosophila. The genes found to be evolving at faster rates in cold-tolerant species were enriched for many of the same functional categories as in our current study including e.g. membrane and transmembrane proteins and immunoglobulins (Parker et al., 2018).

Membrane proteins and lipids are an important determinant of membrane and cuticular permeability at different temperatures, which in turn has an effect on the resistance to desiccation stress in insects (Gibbs, 2002; Stanziano, Sové, Rundle, & Sinclair, 2015). Importantly, there is evidence for a close link between the desiccation stress response and cold tolerance across species and in *Drosophila* in particular, suggesting an overlap in some of the mechanisms involved (Sinclair, Nelson, Nilson, Roberts, & Gibbs, 2007). Moreover, cold-hardy lines of *D. melanogaster* are known to exhibit elevated lipid metabolism, perhaps in order to allow rapid lipid membrane modification (Williams et al., 2016) in different environmental conditions. Furthermore, Pleckstrin homology (PH) domain was one of the functional clusters found in both the current study and in the species comparison of Parker et al., (2018). This domain is a flexible module of 100-120 amino acids which interacts with a variety of different ligands, composing a protein-protein interaction platform (Scheffzek & Welti, 2012). As changes in the membrane lipid biochemistry form an integral part of the cold tolerance response, genes associated with PH may also assist in homeoviscous adaptation i.e. alteration of membrane phospholipid composition to maintain its fluidity at the low temperatures (Sinensky, 1974). Interestingly, the only gene found in all five of our previous studies of cold tolerance and other phenotypes related to it in *D. montana* was *sidestep II* (*side-II*). Unfortunately, there is no information available for the biological processes or molecular functions associated with this gene but *side-II* has protein features including immunoglobulin and immunoglobulin-like domain superfamily (Thurmond et al., 2019) and could hence be involved in the immunological processes during cold response of the flies. Immunoglobulins were also one of the functional clusters enriched among rapidly evolving genes in cold-tolerant species Parker et al. (2018). Insects are known to produce a diverse range of antimicrobial peptides and proteins as part of their immune activity against viruses, bacteria, fungi and parasites (Mylonakis, Podsiadlowski, Muhammed, & Vilcinskas, 2016) and hence immune

responses could be part of the general stress response in cold tolerance (Sinclair, Ferguson, Salehipour-shirazi, & MacMillan, 2013; Ferguson, Heinrichs, & Sinclair, 2016). Consequently, our results indicate that some of the same biochemical processes that are targeted by selection on larger evolutionary scales (i.e. across species), are also involved in local adaptation for different populations within a species, providing a rare bridge between the processes of population differentiation and speciation.

At the chromosomal level, we found an over-representation of loci associated with ecological selection on chromosomes X and 4. It is well-known that X chromosomes can generally evolve quickly due to selection on semi-recessive advantageous loci in the hemizygous sex, and smaller effective population size (Charlesworth, Coyne, & Barton, 1987) and are often most divergent between closely related species (Abbott, Norden, & Hansson, 2017; Ellegren et al., 2012). However, there is no obvious reason to expect faster divergence in chromosome 4. Both the X chromosome and chromosome 4 of *D. montana* are known to harbour several polymorphic inversions (Stone, Guest, & Wilson, 1960; Morales-Hojas, Päällysaho, Vieira, Hoikkala, & Vieira, 2007) and inversions have often been found to vary clinally and to contribute to genomic differentiation throughout clines (e.g. Kolaczkowski et al., 2011; Cheng et al., 2012; Kapun et al., 2016). The reduced recombination within inversions can capture independently advantageous alleles under selection (Kirkpatrick & Barton, 2006). Divergence is often greater in chromosomes carrying inverted regions and non-colinear regions (Lohse, Clarke, Ritchie, & Etges, 2015) and such regions may divergence more quickly during speciation with gene flow. Indeed, here we found that the X chromosome and chromosome 4 always have the highest levels of F_{st} across all populations, as expected if the ecological selection on loci on these chromosomes influenced overall patterns of genomic diversion, perhaps due to hitchiking. Given these findings, further investigation of the known inversion polymorphisms in across *D. montana* populations would be very interesting. Finally, our findings include intriguing result regarding the Crested Butte population which shows reduced genetic variability and a substantial reduction of Tajima's D relative to the other populations. This population occurs at a very high altitude (>2800 m) and also shows reproductive incompatibilities with other populations (Jennings et al., 2014). It may have been bottlenecked during its adaptation to this high altitude, so population expansion or selected sweeps could be prevalent within this population. Whether ecological specialisation is associated with the spread of incompatibilities is an intriguing possibility.

CONCLUSION

Identifying the genetic variation that underlies population divergence and ultimately speciation remains a challenge. Detecting associations between genetic and environmental

differentiation at loci across populations has become a popular approach. Here we apply Bayesian methods to detect such loci across populations of *Drosophila montana,* which is an extraordinarily cold-tolerant *Drosophila* species where we expect strong ecological selection. We identify many genes that are associated with both climate variables and population-level cold-tolerance phenotypes. These genes also overlap with candidate genes form other studies of variation in cold-tolerance in *D. montana*. Our study presents an excellent example of how strong ecological selection can be detected in genome studies, using Bayesian methods to detect local adaptation in combination with studies of ecologically important phenotypes.

Data Availability

Raw reads have been deposited with NCBI under the BioProject accession PRJNA588720.

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REFERENCES

Abbott, J. K., Norden, A. K., & Hansson, B. (2017). Sex chromosome evolution: Historical insights and future perspectives. *Proceedings of the Royal Society B: Biological Sciences*, *284*. doi: 10.1098/rspb.2016.2806

Andersen, J. L., Manenti, T., Sorensen, J. G., MacMillan, H. A., Loeschcke, V., & Overgaard, J. (2015). How to assess Drosophila cold tolerance: Chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. *Functional Ecology, 29*, 55–65. doi: 10.1111/1365-2435.12310

Andrews, S. (2015). FastQC: A quality control tool for high throughput sequence data. Retrieved from http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/

Barnett, D. W., Garrison, E. K., Quinlan, A. R., Strömberg, M. P., & Marth, G. T. (2011). Bamtools: A C++ API and toolkit for analyzing and managing BAM files. *Bioinformatics*, *27*, 1691–1692. doi: 10.1093/bioinformatics/btr174

Barton, N. H., & Gale, K. S. (1993). Genetic analysis of hybrid zones. In R. G. Harrison (Ed.), *Hybrid Zones and the Evolutionary Process.* (pp. 13–45). Oxford, UK: Oxford University Press.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical* Software*, 67*, 1–48. doi: 10.18637/jss.v067.i01

Bauerfeind, S. S., Kellermann, V., Moghadam, N. N., Loeschcke, V., & Fischer, K. (2014). Temperature and photoperiod affect stress resistance traits in *Drosophila melanogaster*. *Physiological Entomology, 39*, 237–246. doi: 10.1111/phen.12068

Bergland, A. O., Behrman, E. L., O'Brien, K. R., Schmidt, P. S., & Petrov, D. (2014). Genomic evidence of rapid and stable adaptive oscillations over seasonal time scales in Drosophila. *PLoS Genetics, 10*, e1004775. doi: 10.1371/journal.pgen.1004775

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics, 30*, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bradbury, D., Smithson, A., & Krauss, S. L. (2013). Signatures of diversifying selection at EST-SSR loci and association with climate in natural Eucalyptus populations. *Molecular Ecology, 22,* 5112–5129. doi: 10.1111/mec.12463

Broad Institute. Picard. Retrieved from http://broadinstitute.github.io/picard/

Carneiro, M., Baird, S. J. E., Afonso, S., Ramirez, E., Tarroso, P., Teotónio, H., … Ferrand, N. (2013). Steep clines within a highly permeable genome across a hybrid zone between two subspecies of the European rabbit. *Molecular Ecology, 22*, 2511–2525. doi: 10.1111/mec.12272

Charlesworth, B., Coyne, J. A., & Barton, N. H. (1987). The relative rates of evolution of sex chromosomes and autosomes. *The American Naturalist, 130*, 113–146.

Chen, J., Källman, T., Ma, X., Gyllenstrand, N., Zaina, G., Morgante, M., … Lascoux, M. (2012). Disentangling the roles of history and local selection in shaping clinal variation of allele frequencies and gene expression in Norway spruce (*Picea abies*). *Genetics, 191*, 865–881. doi: 10.1534/genetics.112.140749

Cruickshank, T. E., & Hahn, M. W. (2014). Reanalysis suggests that genomic islands of speciation are due to reduced diversity, not reduced gene flow. *Molecular Ecology, 23*, 3133–3157. doi: 10.1111/mec.12796

David, R., Gibert, P., Pla, E., Petavy, G., Karan, D., & Moreteau, B. (1998). Cold stress tolerance in *Drosophila*: Analysis of chill coma recovery in *D. melanogaster*. *Journal of Thermal Biology, 23*, 291–299. doi: 10.1016/S0306-4565(98)00020-5

Ellegren, H., Smeds, L., Burri, R., Olason, P. I., Backstrom, N., Kawakami, T., … Wolf, J. B. W. (2012). The genomic landscape of species divergence in *Ficedula* flycatchers. *Nature, 491,* 756–760. doi: 10.1038/nature11584

Endler, J. A. (1973). Gene flow and differentiation. *Science. 179*, 243–250. doi: 10.1126/science.179.4070.243

Endler, J. A. (1977). *Geographic Variation, Speciation, and Clines.* Princeton, NJ: Princeton University Press.

Feder, A. F., Petrov, D. A., & Bergland, A. O. (2012). LDx: Estimation of linkage disequilibrium from high-throughput pooled resequencing data. *PLoS ONE*, *7*. doi: 10.1371/journal.pone.0048588

Ferguson, L. V, Heinrichs, D. E., & Sinclair, B. J. (2016). Paradoxical acclimation responses in the thermal performance of insect immunity. *Oecologia, 181*, 77–85. doi: 10.1007/s00442-015-3529-6

Findsen, A., Andersen, J. L., Calderon, S., & Overgaard, J. (2013). Rapid cold hardening improves recovery of ion homeostasis and chill coma recovery time in the migratory locust, *Locusta migratoria. Journal of Experimental Biology, 216*, 1630–1637. doi: 10.1242/jeb.081141

Findsen, A., Pedersen, T. H., Petersen, A. G., Nielsen, O. B., & Overgaard, J. (2014). Why do insects enter and recover from chill coma? Low temperature and high extracellular potassium compromise muscle function in *Locusta migratoria*. *The Journal of experimental biology, 217*, 1297–1306. doi: 10.1242/jeb.098442

Foll, M., & Gaggiotti, O. E. (2008). A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: A Bayesian perspective. *Genetics*, *180*, 977–993. doi: 10.1534/genetics.108.092221

Foll, M., Gaggiotti, O. E., Daub, J. T., Vatsiou, A., & Excoffier, L. (2014). Widespread signals of convergent adaptation to high altitude in Asia and America. *American Journal of Human Genetics, 95*, 394–407. doi: 10.1016/j.ajhg.2014.09.002

Gautier, M. (2015). Genome-wide scan for adaptive divergence and association with population-specific covariates. *Genetics, 201*, 1555–1579. doi: 10.1534/genetics.115.181453

Gibbs, A. G. (2002). Lipid melting points and cuticular permeability: New insights into an old problem. *Journal of Insect Physiology, 48*, 391–400. doi: 10.1016/s0022-1910(02)00059-8

Gibert, P., Moreteau, B., Petavy, G., Karan, D., & David, J. (2001). Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. *Evolution, 55*,1063–1068. doi: 10.1554/0014-3820(2001)055[1063:cctamc]2.0.co;2

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G., & Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, *25*, 1965–1978. doi: 10.1002/joc.1276

Hijmans, R. E., van Etten, J., Cheng, J., Mattiuzzi, M., Sumner, M., Greenberg, J. A., … Wueest, R. (2016). raster: Geographic Data Analysis and Modeling. Retrieved from: https://CRAN.R-project.org/package=raster

Hivert, V., Leblois, R., Petit, E. J., Gautier, M., & Vitalis, R. (2018). Measuring genetic differentiation from pool-seq data. *Genetics*, *210*, 315–330. doi: 10.1534/genetics.118.300900

Hoekstra, H., Drumm, K., & Nachman, M. (2004). Ecological genetics of adaptive color polymorphism in pocket mice: Geographic variation in selected and neutral genes. *Evolution, 58*, 1329–1341. doi: 10.1111/j.0014-3820.2004.tb01711.x

Jennings, J. H., Mazzi, D., Ritchie, M. G., & Hoikkala, A. (2011). Sexual and postmating reproductive isolation between allopatric *Drosophila montana* populations suggest speciation potential. *BMC Evolutionary Biology*, *11*. doi: 10.1186/1471-2148-11-68

Jennings, J. H., Snook, R. R., & Hoikkala, A. (2014). Reproductive isolation among allopatric *Drosophila montana* populations. *Evolution, 68*, 3095–3108. doi: 10.1111/evo.12535

Kankare, M., Salminen, T., Laiho, A., Vesala, L., & Hoikkala, A. (2010). Changes in gene expression linked with adult reproductive diapause in a northern malt fly species: A candidate gene microarray study. *BMC Ecology*, *10*. doi: 10.1186/1472-6785-10-3

Kankare, M., Parker, D. J., Merisalo, M., Salminen, T. S., & Hoikkala, A. (2016). Transcriptional differences between diapausing and non-diapausing *D. montana* females reared under the same photoperiod and temperature. *PLoS ONE, 11,* e0161852. doi: 10.1371/journal.pone.0161852

Kapun, M., Fabian, D.K., Goudet, J., & Flatt, T. (2016). Genomic evidence for adaptive inversion clines in *Drosophila melanogaster*. *Molecular Biology and Evolution, 33*, 1317–1336. doi: 10.1093/molbev/msw016

Kapun, M., Barrón, M. G., Staubach, F., Vieira, J., Obbard, D. J., Goubert, C., … González, J. (2019). Genomic analysis of European *Drosophila melanogaster* populations on a dense spatial scale reveals longitudinal population structure and continent-wide selection, and unknown DNA viruses. *BioRxiv*. doi: 10.1101/313759

Kauranen, H., Kinnunen, J., Hiillos, A-L., Lankinen, P., Hopkins, D., Wiberg, R. A. W., … Hoikkala, A. (2019). Selection for reproduction under short photoperiods changes diapause-associated traits and induces widespread genomic divergence. *Journal of Experimental Biology*, *222*, jeb205831. doi: 10.1242/jeb.205831

Kellermann, V., Overgaard, J., Hoffmann, A. A., Flojgaard, C., Svenning, J. C., & Loeschcke, V. (2012). Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences of the United States of America, 109*, 16228–16233. doi: 10.1073/pnas.1207553109

Kirkpatrick, M., & Barton, N. (2006). Chromosome inversions, local adaptation and speciation. *Genetics*, *173*, 419–434. doi: 10.1534/genetics.105.047985

Klappert, K., Mazzi, D., Hoikkala, A., & Ritchie, M. G. (2007). Male courtship song and female preference variation between phylogeographically distinct populations of *drosophila montana*. *Evolution*, *61*, 1481–1488. doi: 10.1111/j.1558-5646.2007.00125.x

Kolaczkowski, B., Kern, A. D., Holloway, A. K., & Begun, D. J. (2011). Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. *Genetics*, *187*, 245–260. doi: 10.1534/genetics.110.123059

Lakovaara, S. (1969). Malt as a culture medium for Drosophila species. *Drosophila Information Service, 44*, 128.

Lankinen, P., Tyukmaeva, V. I., & Hoikkala, A. (2013). Northern *Drosophila montana* flies show variation both within and between cline populations in the critical day length evoking reproductive diapause. *Journal of Insect Physiology*, *59*, 745–751. doi: 10.1016/j.jinsphys.2013.05.006

Lê, S., Josse, J., & Husson, F. (2008). FactoMineR: An R package for multivariate analysis. *Journal of Statistical Software*, *25*, 1–18. doi: 10.18637/jss.v025.i01

Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:1303.3997v1 [q-bio.GN] Retrieved from: https://arxiv.org/abs/1303.3997

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., ... The 1000 Genome Project Data Processing Subgroup. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*, 2078–2079. doi: 10.1093/bioinformatics/btp352

Lohse, K., Clarke, M., Ritchie, M. G., & Etges, W. J. (2015). Genome-wide tests for introgression between cactophilic Drosophila implicate a role of inversions during speciation. *Evolution, 69*, 1178–1190. doi: 10.1111/evo.12650

Machado, H. E., Bergland, A. O., O'Brien, K., Behrman, E. L., Schmidt, P. S., & Petrov, D. (2015). Comparative population genomics of latitudinal variation in *Drosophila simulans* and *Drosophila melanogaster*. *Molecular Ecology, 25*, 723–740. doi: 10.1111/mec.13446

MacMillan, H. A., Williams, C. M., Staples, J. F., & Sinclair, B. J. (2012). Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. *Proceedings of the National Academy of Sciences of the United States of America, 109*, 20750–20755. doi: 10.1073/pnas.1212788109

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., ... DePristo, M. (2010). The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research*, *20*, 1297–1303. doi: 10.1101/gr.107524.110

636 Morales-Hojas, R., Päällysaho, S., Vieira, C. P., Hoikkala, A., & Vieira, J. (2007). Comparative polytene chromosome maps of *D. montana* and *D. virilis*. *Chromosoma, 116*, 21–7.doi: 10.1007/s00412-006-0075-3

639 Mylonakis, E., Podsiadlowski, L., Muhammed, M., & Vilcinskas, A. (2016). Diversity, evolution and medical applications of insect antimicrobial peptides. *Philosophical Transactions of the Royal Society B: Biological Sciences, 371*, 20150290. doi: 10.1098/rstb.2015.0290

642 Noor, M. A. F., & Bennett, S. M. (2009). Islands of speciation or mirages in the desert? Examining the role of restricted recombination in maintaining species. *Heredity, 103*, 439–444. doi: 10.1038/hdy.2009.151

645 Parker, D. J., Vesala, L., Ritchie, M. G., Laiho, A., Hoikkala, A., & Kankare, M. (2015). How consistent are the transcriptome changes associated with cold acclimation in two species of the *Drosophila virilis* group? *Heredity*, *115*, 13–21. doi: 10.1038/hdy.2015.6

648 Parker, D. J., Ritchie, M. G., & Kankare, M. (2016). Preparing for Winter: The transcriptomic response associated with different day lengths in *Drosophila montana*. *G3: Genes, Genomes, Genetics, 6*, 1373–1381. doi: 10.1534/g3.116.027870

651 Parker, D. J., Wiberg, R. A. W., Trivedi, U., Tyukmaeva, V. I., Gharbi, K., Butlin, R. K., ... Ritchie, M. G. (2018). Inter- and intra-specific genomic divergence in *Drosophila montana* shows evidence for cold adaptation. *Genome Biology and Evolution*, *10*, 2086–2101.

654 doi: 10.1093/gbe/evy147

Plummer, M., Best, N., Cowles, K., & Vines, K. (2006). CODA: Convergence diagnosis and output analysis for MCMC. *R News*, *6*, 7–11.

657 Paolucci, S., Salis, L., Vermeulen, C. J., Beukeboom, L. W., & van de Zande, L. (2016). QTL analysis of the photoperiodic response and clinal distribution of period alleles in Nasonia vitripennis. *Molecular Ecology, 25*, 4805–4817. doi: 10.1111/mec.13802

660 R Development Core Team. (2019). R: A language and environment for statistical computing. Retrieved from http://www.r-project.org

Ravinet, M., Faria, R., Butlin, R. K., Galindo, J., Bierne, N., Rafajlovic, M., …Westram, A. M.

663 (2017). Interpreting the genomic landscape of speciation: A road map for finding barriers to gene flow. *Journal of Evolutionary Biology, 30*, 1450–1477. doi: 10.1111/jeb.13047

Routtu, J., Mazzi, D., van der Linde, K., Mirol, P., Butlin, R., & Ritchie, M. G. (2007). The extent

666 of variation in male song, wing and genital characters among allopatric *Drosophila montana* populations. *Journal of Evolutionary Biology, 20*, 1591–1601. doi: 10.1111/j.1420-9101.2007.01323.x

669 Scheffzek, K., & Welti, S. (2012). Pleckstrin homology (PH) like domains – versatile modules in protein–protein interaction platforms. *FEBS Letters, 586*, 2662–2673. doi: 10.1016/j.febslet.2012.06.006

672 Schlötterer, C., Tobler, R., Kofler, R., & Nolte, V. (2014). Sequencing pools of individuals — mining genome-wide polymorphism data without big funding. *Nature Reviews Genetics, 15*, 749–763. doi: 10.1038/nrg3803

675 Sinclair, B.J., Ferguson, L. V., Salehipour-shirazi, G., & MacMillan, H. A. (2013). Cross-tolerance and cross-talk in the cold: Relating low temperatures to desiccation and immune stress in insects. *Integrative and Comparative Biology, 53*, 545–556. doi: 10.1093/icb/ict004

678 Sinclair, B. J., Nelson, S., Nilson, T. L., Roberts, S. P., & Gibbs, A. G. (2007). The effect of selection for desiccation resistance on cold tolerance of *Drosophila melanogaster. Physiological Entomology, 32*, 322–327. doi: 10.1111/j.1365-3032.2007.00585.x

681 Sinensky, M. (1974). Homeoviscous adaptation–A homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America, 71*, 522–525. doi: 10.1073/pnas.71.2.522

684 Stanziano, J. R., Sové, R. J., Rundle, H. D., & Sinclair, B. J. (2015). Rapid desiccation hardening changes the cuticular hydrocarbon profile of *Drosophila melanogaster*. *Comparative Biochemistry and Physiology, 180*, 38–42. doi: 10.1016/j.cbpa.2014

687 Stone, W. S., Guest, W. C., & Wilson, F. D. (1960). The evolutionary implications of the cytological polymorphism and phylogeny of the *virilis* group of Drosophila. *Proceedings of the National Academy of Sciences of the United States of America, 46*, 350–361. doi: 10.1073/pnas.46.3.350

690 Takahashi, Y. (2015). Mechanisms and tests for geographic clines in genetic polymorphisms. *Population Ecology. 57*, 355–362.

Throckmorton, L. H. (1969). The *virilis* Species Group. *The Genetics and Biology of Drosophila, 3,*
693 (pp. 227–296).

Thurmond, J., Goodman, J. L., Strelets, V. B., Attrill, H., Gramates, L. S., Marygold, S. J., … The FlyBase Consortium. (2019). FlyBase 2.0: The next generation. *Nucleic Acids Research*, *47,*
696 759–765. doi: 10.1093/nar/gky1003

Tyukmaeva, V. I., Salminen, T. S., Kankare, M., Knott, E. & Hoikkala, A. (2011). Adaptation to a seasonally varying environment: A strong latitudinal cline in reproductive diapause combined
699 with high gene flow in *Drosophila montana*. *Ecology and Evolution, 1,* 160–168. doi: 10.1002/ece3.14

Tyukmaeva, V. I., Lankinen, P., Kinnunen, J., Kauranen, H., & Hoikkala, A. (2020). Latitudinal clines in the timing and temperature-sensitivity of photoperiodic reproductive diapause in *Drosophila montana*. *Ecography, 43*, 1–10. doi: 10.1111/ecog.04892

Vesala, L., & Hoikkala, A. (2011). Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of Drosophila montana. *Journal of Insect Physiology, 57,* 46–51. doi: 10.1016/j.jinsphys.2010.09.007

Vesala, L., Salminen, T. S., Kostal, V., Zahradnickova, H., & Hoikkala, A. (2012). *Myo*-inositol as a main metabolite in overwintering flies: Seasonal metabolomic profiles and cold stress tolerance in a northern drosophilid fly. *Journal of Experimental Biology, 215*, 2891–2897. doi: 10.1242/jeb.069948

Vesala, L., Salminen, T. S., Laiho, A., Hoikkala, A., & Kankare M. (2012). Cold tolerance and cold-induced modulation of gene expression in two Drosophila virilis group species with different distributions. *Insect Molecular Biology, 21*, 107–118. doi: 10.1111/j.1365-2583.2011.01119.x

Vigoder, F. M., Parker, D. J., Cook, N., Tournière, O., Sneddon, T., & Ritchie, M. G. (2016). Inducing Cold-Sensitivity in the Frigophilic Fly *Drosophila montana* by RNAi. *PLoS ONE, 11*, 1–9. doi: 10.1371/journal.pone.0165724

Vines, T. H., Dalziel, A. C., Albert, A.Y. K., Veen, T., Schulte, P. M., & Schluter, D. (2016). Cline coupling and uncoupling in a stickleback hybrid zone. *Evolution. 70,* 1023–1038. doi: 10.1111/evo.12917

de Villemereuil, P., & Gaggiotti, O. E. (2015). A new FST-based method to uncover local adaptation using environmental variables. *Methods in Ecology and Evolution, 6*, 1248–1258. doi: 10.1111/2041-210X.12418

Wilder, S. M., Mayntz, D., Toft, S., Rypstra, A. L., Pilati, A., & Vanni, M. J. (2010). Intraspecific variation in prey quality: A comparison of nutrient presence in prey and nutrient extraction by predators. *Oikos, 119*, 350–358. doi: 10.1111/j.1600-0706.2009.17819.x

Williams, C. M., McCue, M. D., Sunny, N. E., Szejner-Sigal, A., Morgan, T. J., Allison, D. B., & Hahn, D. A. (2016). Cold adaptation increases rates of nutrient flow and metabolic plasticity during cold exposure in Drosophila melanogaster. *Proceedings of the Royal Society B: Biological Sciences, 283*, 20161317. doi: 10.1098/rspb.2016.1317

Wolf, J. B. W., & Ellegren, H. (2016). Making sense of genomic islands of differentiation in light of speciation. *Nature Reviews Genetics*, *18*, 87–100. doi: 10.1038/nrg.2016.133

Tables, Figures, and Figure Legends

Table 1. The sources of population genomic samples (coordinates and the name of the nearest town), altitude of the sampling site, the year in which sampling was performed, and the number of males and females sampled (M/F) for each pool.

Source	Sampling Site	Year	M/F
USA, Alaska	Seward 60°9'N; 149°27'W Altitude 35 m	2013	30/20
Canada, British Columbia	Terrace 54°27'N; 128°34'W Altitude 217 m	2014	22/27
USA, Washington	Ashford, 46°45'N; 121°57'W Altitude 573 m	2013	16/34
USA, Colorado	Crested Butte 38°54'N; 106°57'W Altitude 2900 m	2013	36/13
Finland	Oulanka 66°40'N; 29°20'E Altitude 337	2013	25/25
Finland	Korpilahti 62°20'N; 25°34'E Altitude 133	2013	27/23

Table 2. Number of SNPs with a q-value < 0.05 on each linkage group (chromosomes) and each environmental variable (see Methods).

Environmental Variable	**LG2**	**LG3**	**LG4**	**LG5**	**X**
PC1	561	485	1,432	498	1,528
PC2	1,639	1,191	2,480	1,297	1,861
CTmin	502	399	1,383	384	1,272
CCRT	360	312	1,252	316	1,228

744 **Table 3.** Functional gene enrichment analyses of the genes withing 10kb of candidate SNPs. Table shows if an annotation term was significantly enriched among SNPs for each cold tolerance and climatic variable as well as for the subset of genes in common for all variables (see detailed
747 information in table S4).

CLUSTERS	**All four variables**	**CCRT**	**CTmin**	**PC 1**	**PC 2**
Membrane		x		x	
Transmembrane		x		x	
Immunoglobulins		x	x		x
Carbohydrate		x			
Glycoside hydrolase	x	x			
HAD hydrolase	x	x	x	x	x
ATPase activity		x			
ABC transport		x			
Phosphate		x	x		
Rho/Pleckstrin/ Guanide			x	x	x
Nucleotide binding			x	x	x
Lipid metabolism/Lipase			x		x
Ion channels & transport				x	
SH3 domain				x	
Cation efflux protein				x	
WD40 repeat				x	
Metal binding				x	

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753 **Table 4.** Mean (SD) F_{st} values for each linkage group (chromosome) and population

Chromosome	Oulanka	Korpilahti	Ashford	Seward	Crested Butte	Terrace
LG2	0.12	0.12	0.10	0.10	0.15	0.11
LG3	0.11	0.11	0.09	0.09	0.15	0.10
LG4	0.15	0.15	0.14	0.13	0.19	0.13
LG5	0.12	0.13	0.11	0.11	0.16	0.11
X	0.16	0.16	0.14	0.15	0.19	0.14

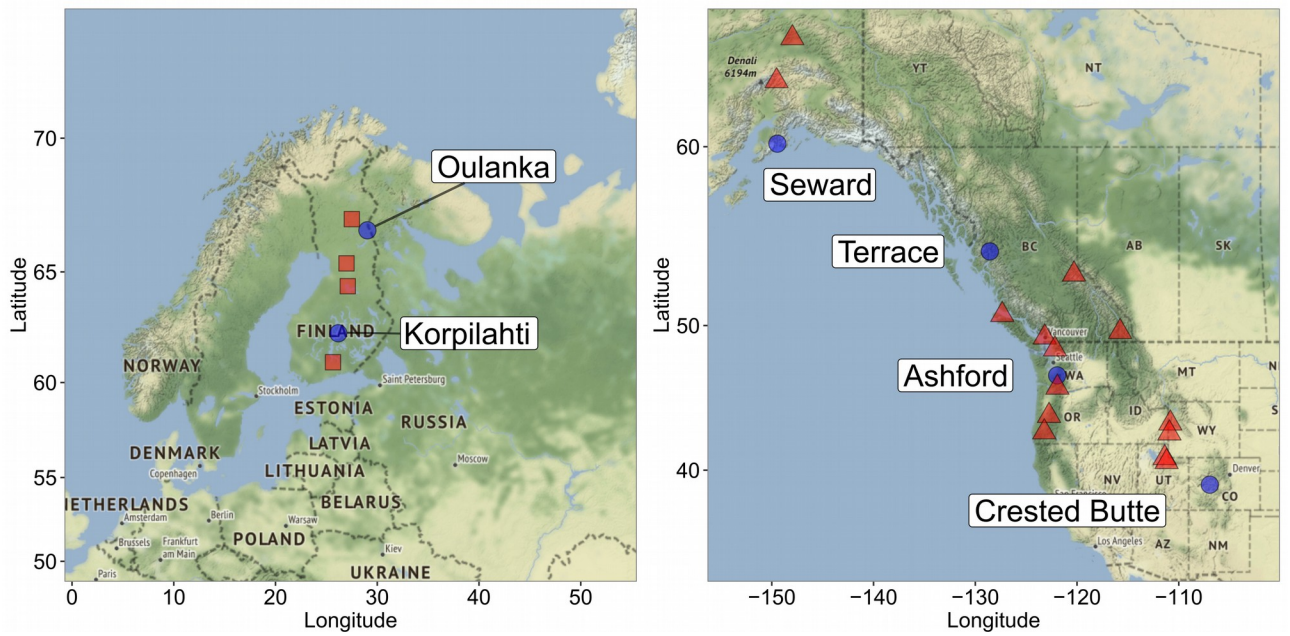
Figure 1. Maps of **A)** North America and **B)** Finland showing the locations of all populations sampled. Labelled, blue circles give the locations of populations sampled for phenotyping and sequencing.

Figure 2. Population phenotypes. **A)** and **B)** give the variation in CTmin across populations and latitude respectively. Solid and dashed lines show the predicted values from the best model (see Results) for males and females respectively. **C)** and **D)** show the variation in CCRT across populations and latitude. The trend line in **D)** gives the predicted fits from the best model (see Results), although points are plotted separately for males and females, the best model only included latitude as a covariate.

Figure 3. Principle components results. **A)** The distribution of all populations along the two fires PC axes. Blue circles give the populations that have been pool-sequenced for this study, red triangles and squares give other North American and Finnish populations respectively. **B)** The eigenvalues of all PCs as well as the cumulative variance explained (inset). Dashed horizontal lines give the threshholds of an eigenvalue of 1 and 98% of variance explained (inset). **C)** and **D)** give PCs 1 and 2 as a function of latitude and altitude.

Figure 4. Overlap of genes within 10kb of top SNPs from BayeScEnv analyses for PC1, PC2 CTmin, and CCRT. The total set sizes are given by the bars in the bottom left. The overlaps are depicted by points connected by lines along the *x*-axis and the height of the bars indicate the size of each set.

Figure 1



780 **Figure 2**

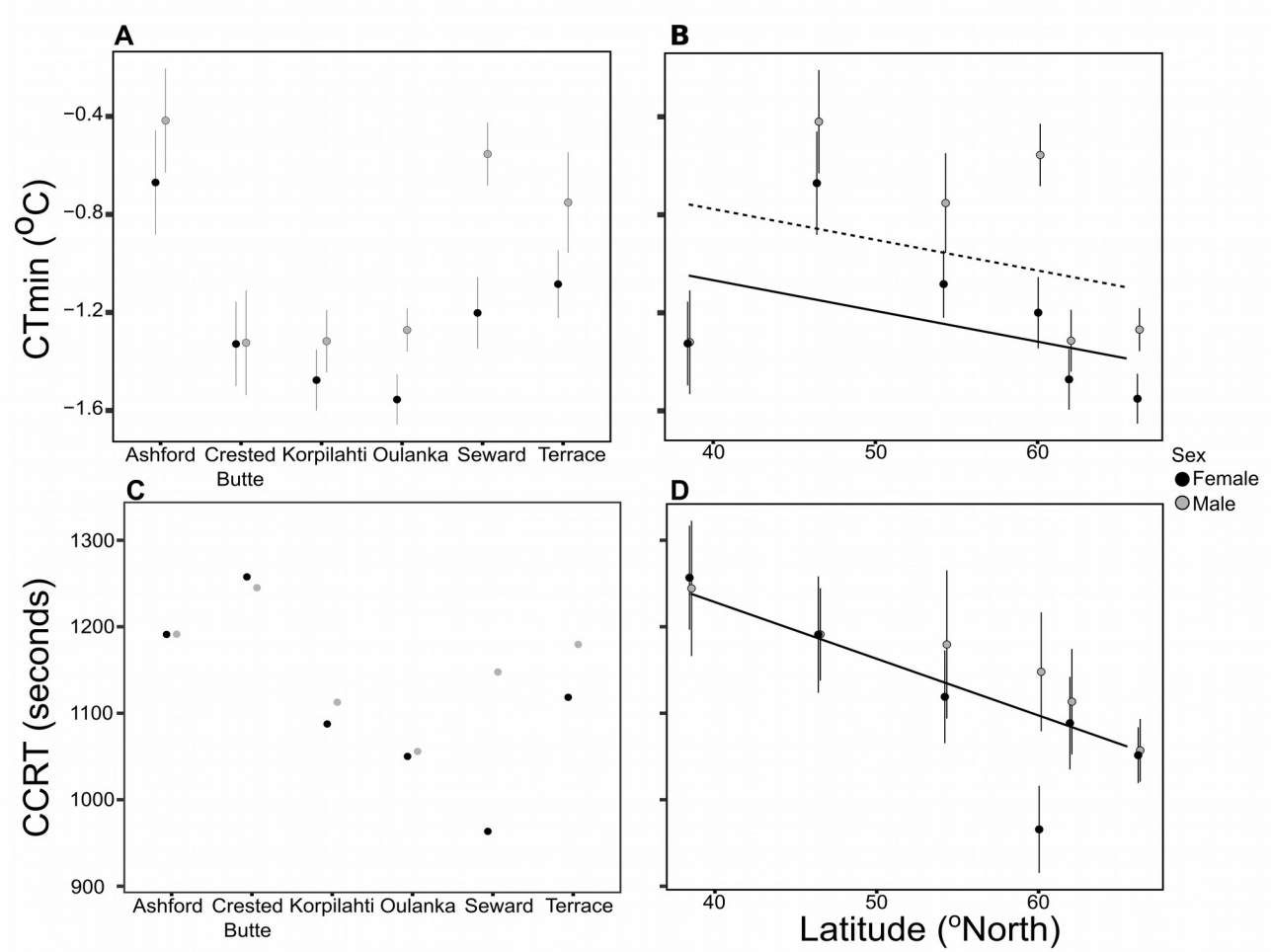


Figure 3.

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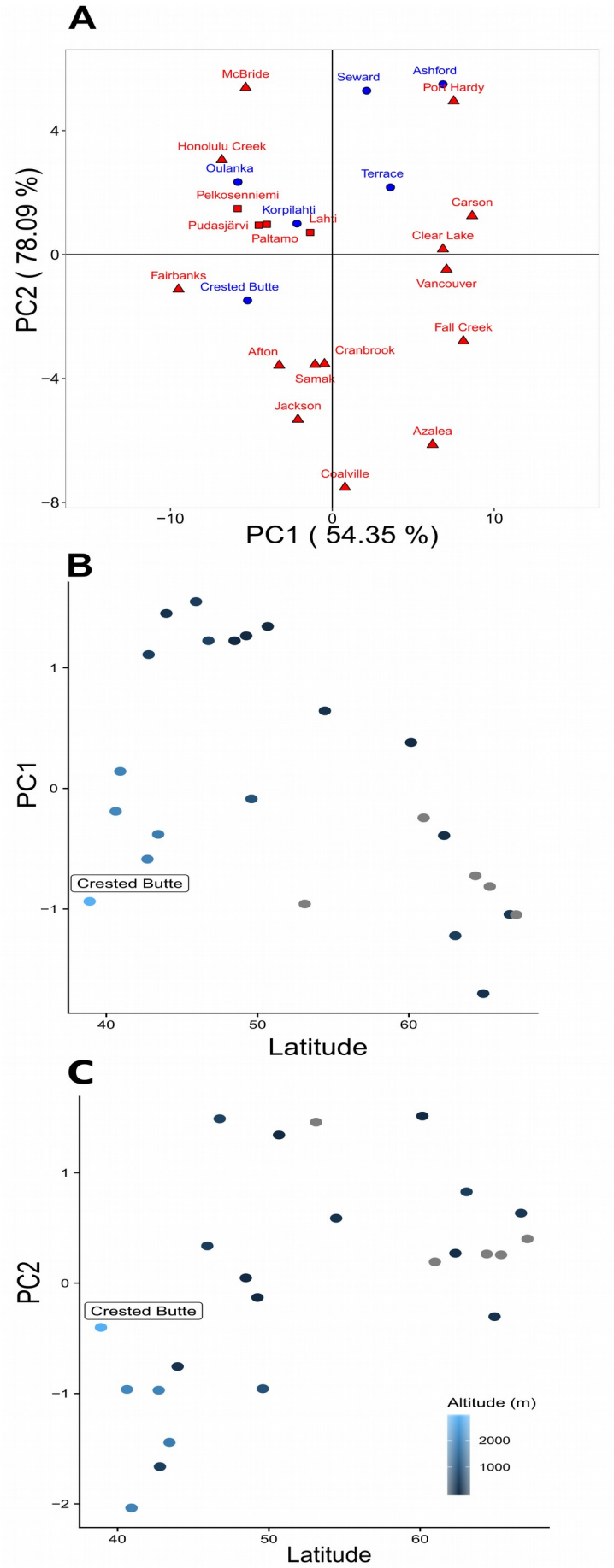

Figure 4.