Genome sequencing and morphometric analysis of alpine bumblebees experiencing climate change reveal ongoing phenotypic shifts and a lack of population structure

Running title: Genome variation in alpine bumblebees

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ABSTRACT

Populations of the bumblebees *Bombus sylvicola* and *Bombus balteatus* in Colorado have experienced decreases in tongue length, a trait important for plant-pollinator mutualisms, in the last six decades. It is hypothesized that this reflects selection exerted by changes in floral composition under climate change. Here we combine extensive morphometric and population genomic data to investigate population structure, whether morphological change is ongoing, and the genetic basis of morphological change. We generate highly-contiguous genome assemblies of both species using long-read sequencing. We then perform whole-genome sequencing and morphometric measurements of 580 samples of these species from seven high-altitude localities. Out of 281 samples originally identified as *B. sylvicola*, 67 formed a separate genetic cluster comprising the newly-discovered cryptic species *B. incognitus*. However, there is very little additional genetic substructure, suggesting that gene flow occurs readily between mountains. We find a significant decrease in tongue length between bees collected between 2008-2014 and in 2017, indicating that morphological shifts are ongoing. We did not discover any genetic associations with tongue length, but a SNP related to production of a proteolytic digestive enzyme is implicated in body size variation. We identify evidence of covariance between kinship and both tongue length and body size, which is suggestive of a genetic component of these traits, although it is not possible to rule out shared environmental effects between colonies. Our results provide evidence for ongoing evolution of a morphological trait important for pollination and indicate that this trait likely has a complex genetic and environmental basis.

Keywords: genome-wide complex trait analysis, plant-pollinator interaction, population structure, genome assembly, population genomics, global warming.

INTRODUCTION

The importance of pollinators for ecology and the maintenance of biodiversity is widely recognized (Goulson, Nicholls, Botías, & Rotheray, 2015). Bumblebees are large-bodied, mainly cold-adapted species with broad significance for agricultural production and plant-pollinator networks in the wild (Goulson, 2003). Many bumblebee species are experiencing range shifts or declines due to climate change or habitat loss (Cameron et al., 2011;

Cameron & Sadd, 2020; Goulson, Lye, & Darvill, 2008; Soroye, Newbold, & Kerr, 2020). In particular, a general worldwide trend for species to shift to higher elevations and latitudes has been documented, with losses occurring at a faster rate than expansions (Kerr et al., 2015; Marshall et al., 2020). Arctic and alpine species are particularly threatened by these trends.

In addition to range shifts there is also evidence that the morphology of certain populations of bumblebees has changed in recent decades. Miller-Struttmann et al. (Miller-Struttmann et al., 2015) compared morphometric measurements of contemporary samples and historical specimens of the species *Bombus balteatus* and *Bombus sylvicola* from the same high-altitude localities in Colorado. These species historically comprise the majority of bumblebees in these locations. They reported a significant decrease in tongue length in both species in a period between 1966 and 2014. The cause of this was inferred to be a general decrease in floral abundance resulting from warmer temperatures and dryer soils. These conditions favor more generalist foraging that is performed more optimally by bees with shorter tongues (Goulson, Hanley, Darvill, Ellis, & Knight, 2005). Continued monitoring of these populations is important to understand the ongoing effects of climate change, but it is unknown whether the trend toward shorter tongues has continued in more recent years.

One explanation for the observed morphological shifts in *B. balteatus* and *B. sylvicola* in Colorado is that natural selection for shorter tongues has occurred (Miller-Struttmann et al., 2015). However, it is also possible that the observed changes are due to phenotypic plasticity. It is unknown whether this trait has a strong genetic component that could be acted on by natural selection. Uncovering the size of the genetic contribution to this trait and the number and identity of genetic loci involved is an important step towards understanding the underlying cause of the morphological changes.

The genetic structure of these populations is also relevant to how they evolve under climate change (Pauls, Nowak, Bálint, & Pfenninger, 2013). It is also important to understand population structure in order to interpret genotype-phenotype correlations and cause of morphological shifts. Importantly, using population resequencing, we have identified the

presence of a previously-undetected cryptic species, which we called *Bombus incognitus* (Christmas, submitted). This species is identified morphologically as *B. sylvicola* but forms a distinct genetic cluster. It is therefore possible that morphological changes in the combined populations of these species could be brought about by changes in the relative abundance of each species, although this has not previously been investigated. Additional population structure within species could lead to a similar effect, particularly if genetic structure associated with morphology exists. An understanding of spatial connectivity between populations in different locations is also needed to predict how they will evolve under climate change and to define the conservation value of subpopulations (Pauls et al., 2013; Razgour et al., 2019).

The aim of this study was to produce high-quality annotated genomes of the bumblebee species *B. balteatus* and *B. sylvicola* and use them to assess whole-genome variation and its connection to geographical and morphological variation in the populations of these species previously studied by (Miller-Struttmann et al., 2015). We constructed genome assemblies based on long-read Oxford Nanopore sequencing. We then performed population re-sequencing of 580 samples identified morphologically as these species in Colorado in seven high-altitude localities spread across 150 km. We studied levels of genetic variation, geographical structure and patterns of relatedness between geographical areas in *B. balteatus*, *B. sylvicola*, and the recently-revealed cryptic species *B. incognitus* (Christmas, submitted), which was also present in our dataset. We then analyzed morphological variation in tongue length and body size in context of previous studies (Miller-Struttmann et al., 2015) in order to determine whether the trend towards decreasing tongue length is ongoing. We also performed a genome-wide analysis of the genetic basis of morphological variation using both genome-wide association studies (GWAS) and genome-wide complex trait analysis (GCTA) (Yang, Lee, Goddard, & Visscher, 2011). The results are informative regarding the degree of connectivity between bumblebee populations, the genetic basis of morphological variation, and the mechanisms of morphological evolution. They also indicate a continuing impact of climate change on the populations.

MATERIALS AND METHODS

Study system

127 The species *B. balteatus* and *B. sylvicola* belong to two subgenera of bumblebees
128 (Alpinobombus and Pyrobombus respectively) that are common in alpine and arctic
129 environments. Both species are closely related to populations in Eurasia that different
130 studies have considered either species or subspecies. *Bombus sylvicola* in North America is
131 closely related to *B. lapponicus* in Eurasia and a recent study classifies it as a subspecies of *B.*
132 *lapponicus* rather than a separate species (Martinet et al., 2019). Similarly, two subspecies
133 of *B. balteatus* have been described previously, restricted respectively to North America and
134 Eurasia, but recent study split these populations into two separate species: North American
135 *B. kirbiellus* and Eurasian *B. balteatus* (Williams et al., 2019). Here we maintain the names *B.*
136 *balteatus* and *B. sylvicola* of our species of interest for consistency with previous ecological
137 studies that we are directly comparing here (Geib, Strange, & Galen, 2015; Miller-
138 Struttmann et al., 2015) and ambiguities regarding species/subspecies status.
139
140 **Sample collection**
141 We collected samples identified as *B. balteatus* and *B. sylvicola* during the summer of 2017.
142 Bumblebees were collected on seven different mountains in Colorado, USA. The existence
143 of *B. incognitus* was unknown to us when the bees were sampled and a large fraction of
144 bees identified as *B. sylvicola* were subsequently assigned to *B. incognitus* on the basis of
145 genetic analysis (Christmas, submitted). Three of these mountains (Mount Evans
146 (39.583883, -105.63845), Niwot Ridge (40.05945, -105.616667), and Pennsylvania Mountain
147 (39.263383, -106.142733)) were also sampled previously by (Miller-Struttmann et al., 2015).
148 Four additional mountains were sampled here (Boreas Mountain (39.419375, -105.95441),
149 Mount Democrat (39.33721, -106.12581), Horseshoe Mountain (39.204605, -106.181085)
150 and Quail Mountain (39.018158, -106.404877)) but were not sampled in the previous study.
151 Each of the mountain locations comprised several neighboring sampling sites separated by
152 short distances and altitudes (full details in supplementary tables S1, S2).
153
154 We collected samples of foraging worker bees from each site using sweeping hand nets. We
155 kept samples in Falcon tubes in cool boxes for transport. Species assignment was performed
156 by observing standard morphological characters. We dissected each sample after placing it
157 at -20°C for approximately 10 minutes. We stored thoraces in 95% ethanol prior to DNA
158 extraction whereas the heads were stored for morphometric analysis.

159

160 **Morphological measurements**

161 We measured intertegular distance (ID) on all of the samples of *B. sylvicola, B. incognitus*
162 and *B. balteatus* using scaled photographs of individual bee heads processed by ImageJ
163 ("ImageJ," n.d.). We characterized the diagnostic traits visible on each head, such as the
164 shape of the malar space, the pile color between and antenna and above the ocelli, the size,
165 color and location of the ocelli relative to the supraorbital line, and the presence of black
166 pile on the scutellum. We then dissected the prementum and glossa from the head, which
167 were then mounted and measured by photographing and analysis in ImageJ ("ImageJ," n.d.).
168 None of the morphological characters measured could be used to distinguish between *B.*
169 *incognitus* and *B. sylvicola*.

170

171 **Genome sequencing and assembly**

172 We used the same sequencing strategy to produce high-quality genome assemblies for both
173 *B. sylvicola* and *B. balteatus*. This involved combining data from Oxford Nanopore (ONT) and
174 10x Genomics Chromium technologies. In both cases, we used DNA extracted from male
175 bees sampled on Niwot Ridge and stored in 95% ethanol prior to DNA extraction. DNA from
176 a single bee was used for each assembly. We performed DNA extraction using a salt-
177 isopropanol extraction followed by size selection using magnetic bead purification to
178 remove fragments < 1 kbp. ONT sequencing was performed using the MinION instrument.
179 For *B. sylvicola*, we used two R9.4 flowcells and the RAD004 kit starting with 3-400 ng DNA
180 per run. This resulted in a total of 9.4 Gbp of sequence data, in 2.5 million reads with a
181 mean length of 3.7 kbp. For *B. balteatus*, we used 1µg DNA in a modified LSK-108 one-pot
182 protocol. 1.75 µl NEB FFPE buffer, 1.75 µl NEB Ultra-II End Prep buffer and 1 µl FFPE repair
183 mix was added to 24 µl DNA and incubated at 20°C for 30 minutes. 2 µl End Prep enzyme
184 mix was then added for blunting, phosphorylation and A-tailing (20 min. 20°C, 10 min. 65°C,
185 10 min. 70°C). AMX (20 µl), ligation enhancer (1µl) and Ultra-II ligase (40 µl) was added for
186 ligation at room temperature for 30 minutes. Clean-up and flowcell loading was done
187 following the standard ONT LSK-108 protocol and the library was sequenced on one R9.4
188 flowcell. This resulted in 23.3 Gpb of sequence data, in 3.9 million reads with a mean length
189 of 5.9 kbp.

190

191 We assembled the ONT reads using the following process: We first used downpore for
192 adaptor trimming and splitting chimeric reads. We then assembled the trimmed reads using
193 wtdbg2 (Ruan & Li, 2020) on default settings. We then ran two rounds of the consensus
194 module Racon and contig improvements using medaka v.0.4, removing contigs of length <
195 20 kbp. We used Illumina short-read data from the population sequencing to perform two
196 rounds of Pilon in order to improve sequence accuracy, particularly around indels.
197
198 We also sequenced the same sample of each species using 10x Genomics Chromium. A 10x
199 GEM library was constructed according to the manufacturer's protocols. We quantitated
200 each library using qPCR and sequenced them together on a single lane of HiSeq 2500 using
201 the HiSeq Rapid SBS sequencing kit version 2, resulting in 150 bp paired-end sequences. The
202 linked reads were mapped to the assembly using Longranger v.2.1.4. We then ran Tigmint
203 v1.1.2 to correct errors in the assembly detected by the read mappings. We removed
204 contigs containing multiple mitochondrial genes identified using BLAST+ v2.9.0. We also
205 removed all contigs shorter than 10 kbp.
206
207 **Genome analysis**
208 BUSCO v3.0.2b (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015) was used to
209 assess the assembly completeness using the hymenoptera_odb9 lineage set and species *B.*
210 *impatiens*. We ran RepeatMasker v.4.1.0 on each genome assembly to characterize genome
211 repeat content, including interspersed repeats and low complexity sequences. It has been
212 shown that bumblebees have a stable 18 chromosome karyotype across all subgenera (Sun
213 et al., 2020). We therefore performed whole-genome synteny alignments between the *B.*
214 *terrestris* chromosome-level genome assembly (Sadd et al., 2015) (downloaded from NCBI;
215 BioProject PRJNA45869) and our assemblies using Satsuma v.3 (Grabherr et al., 2010) to
216 arrange the contigs from our assemblies onto 18 pseudochromosomes.
217
218 **RNAseq & Annotation**
219 We performed RNAseq on a single lane of Illumina HiSeq 2500 using samples from four
220 tissues from both *B. syvicola* and *B. balteatus* (abdomen, head, legs and thorax). This
221 generated 44 Gbp of reads for *B. syvicola* and 42 Gbp for *B. balteatus.* We performed quality
222 control of the data using FastQC and read trimming using fastp (v. 0.20.0). We next

223 generated a guided assembly using Hisat2 and Stringtie (v. 2.0). We also performed a *de*
224 *novo* assembly for reads from each of the four tissues using Trinity (v. 2.0.4). We then
225 generated a species-specific repeat library using the RepeatModeller package. We used
226 RepeatMasker to identify the location of repeats from this library in the genomes.
227
228 We used the MAKER package to compute gene builds. As protein evidence, we downloaded
229 curated protein sequences from the Uniprot Swiss-prot database (561,356 proteins,
230 downloaded 11/2019). An evidence-guided gene build was computed by MAKER
231 constructing gene models from these reference proteins and from the transcript sequences
232 aligned to the genome assembly.
233
234 **Population sequencing, read mapping and variant calling**
235 We extracted DNA from the thoraces of worker bees of the species *B. sylvicola, B. incognitus*
236 and *B. balteatus* collected from across the seven sampling sites using the Qiagen Blood and
237 Tissue kit. We prepared libraries using the Nextera Flex kit and performed sequencing on
238 the Illumina HiSeq X to produce 2 x 150 bp reads. We mapped reads to the appropriate
239 reference genome (*B. sylvicola* for *B. sylvicola* and *B. incognitus* samples; *B. balteatus* for *B.*
240 *balteatus* samples) using the mem algorithm in BWA (H. Li & Durbin, 2009). We performed
241 sorting and indexing of the resultant bam files using samtools (Heng Li et al., 2009) and
242 marked duplicate reads using Picard. We used the Genome Analysis Toolkit (GATK)
243 according to best practices to call variants (McKenna et al., 2010). We first ran
244 HaplotypeCaller on the bam file of each sample to generate a gVCF file for each sample. We
245 then used GenomicsDBImport followed by GenotypeGVCFs to call variants for all *B.*
246 *balteatus* samples together and all *B. sylvicola* and *B. incognitus* samples together. We used
247 thresholds recommended by GATK best practices to filter SNPs using VariantFiltration tool.
248 Only biallelic SNPs were considered for downstream analysis.
249
250 **Population structure and relatedness**
251 We thinned the VCF files to 1 SNP every 10 kbp using the --thin flag in vcftools v.0.1.16 in
252 order to more efficiently assess population structure and relatedness. We calculated p-
253 distance matrices for *B. balteatus* samples and *B. sylvicola* and *B. incognitus* samples
254 combined from the thinned vcf files using VCF2Dis (https://github.com/BGI-

shenzhen/VCF2Dis) and then constructed neighbor-joining trees using the 'ape' package in R v4.0.2. We converted the thinned vcf files to plink format and performed principal components analyses using the 'adegenet' package in R v4.0.2. We used admixture v1.3.0 (Alexander, Novembre, & Lange, 2009) to assess the most likely number of genetic clusters within each species. We ran admixture for K=1 through to 10, with 10 iterations per K value and used the calculated cross-validation (cv) errors to assess the most suitable values of K, with low cv-error indicating higher support.

We estimated the pairwise kinship coefficient between all samples using the method described in (Manichaikul et al., 2010) implemented in the --relatedness2 option of vcftools v0.1.16 (Danecek et al., 2011). Worker bees from the same bumblebee colony are expected to have a relatedness of 0.75 under the assumption of monandry, which is reasonable for the study species (Estoup, Scholl, Pouvreau, & Solignac, 1995). The kinship coefficient (φ) predicted from this relationship is $\frac{3}{8}$ (the probability that two alleles sampled at random from two individuals are identical by descent). We estimated the lower cutoff for the inference criteria to infer this level of relatedness using powers of two as described in (Manichaikul et al., 2010), providing an estimate of $\frac{3}{2^{7/2}}$.

Statistical morphometric analysis

We combined the phenotypic measurements made in this study with those from previous collections published in (Miller-Struttmann et al., 2015). These measurements are ID, glossa length, prementum length, and total tongue length (equal to glossa length plus prementum length). This gave us three time periods to compare amongst: '2017' (samples collected for this study), '2012-2014', and '1966-1980' (both sets published in (Miller-Struttmann et al., 2015)). We performed statistical analysis of variation in glossa length, prementum length, and ID in R v4.0.2. We investigated correlations between all traits for each species in the 2017 set using the 'cor.test' function, which calculates a Pearson's product moment correlation coefficient. For glossa length, we calculated least-squares means (ls-means) to account for body size (represented by ID) and compared between species, geographic locations and years of sample collection using the 'lsmeans' function. We analyzed variation in ID between species, geographic locations and years of sample collection using analysis of

286 variance ('aov' function) and assessed significance of comparisons using Tukey's honestly
287 significant difference (HSD) post hoc test ('TukeyHSD' function).
288
289 **Genome-wide association study**
290 We carried out mixed linear model-based association analyses, implemented in GCTA-
291 MLMA (Yang et al., 2011; Yang, Zaitlen, Goddard, Visscher, & Price, 2014), to identify
292 correlations between allele frequencies and trait variation across the genome for *B.*
293 *balteatus* and *B. sylvicola* samples separately (*B. incognitus* samples were left out of this
294 analysis due to too few samples). First, we created a genetic relationship matrix (GRM) from
295 the genome wide SNPs using the --make-grm function in GCTA. We removed one sample
296 from each pair of sisters by setting a relatedness cut-off of 0.67 using the '--grm-cutoff' flag.
297 We ran the analyses for three traits per species:ID, glossa length, and the residuals from a
298 linear regression of glossa~ID in order to control for body size. The GRM was included as a
299 polygenic/random effect. The analysis assumes phenotypes are normally distributed and so
300 samples were removed in the tails of the distributions to meet this assumption for all traits
301 except ID in *B. sylvicola*, which was normally distributed. We assessed divergence of p-
302 values from neutral expectations using QQ-plots and carried out Bonferroni correction of p-
303 values using the 'p.adjust' function in R v4.0.2 to assess genome-wide significance.
304
305 **Genome-wide complex trait analysis**
306 We used GCTA (Yang et al., 2011) to perform genome-wide complex trait analysis. We
307 estimated the phenotypic variance explained by the genome-wide SNPs using genome-
308 based restricted maximum likelihood (GREML). The analysis was performed separately for
309 each species on measures of ID, glossa length, and glossa length with ID included as a
310 covariate to control for body size. We ran each analysis twice, once using a GRM based on
311 all samples and a second time with the same GRM produced above where one from each
312 pair of sisters was removed. We calculated 95% confidence intervals around each estimate
313 by multiplying the standard error by 1.96. We used R functions provided in (Wang & Xu,
314 2019) as well as the GCTA-GREML power calculator (Visscher et al., 2014) to perform power
315 analysis and estimate the effect sizes we can expect to detect with our population datasets.
316
317 **RESULTS**

318 **Generation of highly-contiguous genome assemblies of *B. sylvicola* and *B. balteatus***
319 We generated *de novo* genome assemblies for the species *B. sylvicola* and *B. balteatus* using
320 Oxford Nanopore (ONT) long-read and 10x Chromium linked-read sequencing. Assembly
321 statistics for BBAL_1.0 (*B. balteatus*) and BSYL_1.0 (*B. sylvicola*) are presented in table 1,
322 which includes statistics for the assemblies of *B. terrestris* (BTER_1.0) and *B. impatiens*
323 (BIMP_2.2) for comparison. Total assembly sizes of 250.1 and 252.1 Mbp for *B. balteatus*
324 and *B. sylvicola* respectively are both above the average assembly size of *Bombus* genomes
325 assembled to date of 247.9 Mbp (22 genomes, range 229.8 – 282.1 Mbp) (Heraghty et al.,
326 2020; Sadd et al., 2015; Sun et al., 2020). Contig N50s of 8.60 and 3.02 for BBAL_1.0 and
327 BSYL_1.0 respectively are the longest and third longest contig N50s of all published *Bombus*
328 assemblies, demonstrating these to be highly contiguous assemblies. Megabase-scale contig
329 N50 are typical of long-read assemblies, such as the ones presented here and in (Heraghty
330 et al., 2020), and are significantly more contiguous than those based on short-read
331 sequencing technologies such as BTER_1.0 and BIMP_2.2.
332
333 High genome completeness and accuracy is also reflected in the BUSCO analysis, with scores
334 of 99.0% (98.7% single copy, 0.3% duplicated, 0.5% fragmented, 0.5% missing) and 98.2%
335 (97.9% single copy, 0.3% duplicated, 0.4% fragmented, 1.4% missing) reported for BBAL_1.0
336 and BSYL_1.0 respectively. Whole genome alignments to the *B. terrestris* assembly (Sadd et
337 al., 2015) resulted in 93.9% and 91.1% of the *B. balteatus* and *B. sylvicola* genomes being
338 placed on 18 pseudochromosomes. Our annotation pipeline annotated 11,711 and 11,585
339 genes in BBAL_1.0 and BSYL_1.0 respectively. These are highly comparable to the 11,874
340 genes in the *B. terrestris* gene set (v. 1.0), but lower than the 12,728 genes in the *B.*
341 *impatiens* gene set (v. 2.1). Gene annotation of the *Bombus* assemblies presented in
342 (Heraghty et al., 2020; Sun et al., 2020) also resulted in a greater number of genes
343 annotated, ranging from 13,325 to 16,970 genes. BUSCO analyses of the annotated genes in
344 our assemblies do suggest that we are missing some genes from our annotations (complete
345 BUSCO genes in BBAL_01 and BSYL_01 respectively: 86.6% and 86.9%). Repeat content of
346 our assemblies is comparable to that of other *Bombus* assemblies, with 17.11% of BBAL_1.0
347 and 17.85% of BSYL_1.0 identified as repetitive by RepeatMasker (full details of repeat
348 classes in supplementary table S3). These proportions are comparable to the repeat content
349 inferred in the *B. terrestris* and *B. impatiens* genomes.

Population resequencing indicates three species clusters, with little population structure within species

The locations of sample collections, which comprise 580 samples from across seven localities in the Rocky Mountains, CO, USA, are presented in Fig. 1 (full details in table S1, S2). These samples were initially identified morphologically as *B. balteatus* and *B. sylvicola*. However, neigbor-joining trees produced from samples of both groups of samples indicate that the *B. balteatus* samples form a single cluster whereas the *B. sylvicola* samples form two clusters (Fig 1). By comparison with an additional dataset (Martinet et al., 2019) we previously inferred the largest cluster to be comprised of *B. sylvicola* samples whereas the second cluster is comprised of the cryptic species *B. incognitus* (Christmas, submitted). Among samples originally designated as *B. sylvicola,* 24% (n=67) are identified in the separate cluster of *B. incognitus* whereas the remaining 76% (n=214) are *B. sylvicola. Bombus balteatus* (n=299) was the most commonly observed species across all localities, followed by *B. sylvicola* (n=214) and *B. incognitus* (n=67). The proportions vary between localities (table S1). *Bombus incognitus* samples were collected on six out of the seven mountains, whereas *B. balteatus* and *B. sylvicola* were found on all seven.

We calculated a pairwise kinship coefficient between samples to infer degrees of relatedness (Danecek et al., 2011; Manichaikul et al., 2010). We inferred a set of samples that were likely to be nestmates. In haplodiploids such as bumblebees, females with the same maternal and paternal parents have a relatedness of 0.75. This is likely to be the case for all workers from the same colony in these species, under the realistic assumption that each colony is headed by a single monandrous female queen (Estoup et al., 1995). Those identified as sisters from the same single-mated queen from the same colony were always collected foraging on the same mountain and at the same or immediately neighboring site (Table S1). This suggests that foraging bees were generally found in the immediate vicinity of their nests.

We investigated the presence of population genetic structure within each species using several methods. Neighbor-joining trees (Fig. 1) do not reveal the presence of any substructure related to geography for *B. balteatus* or *B. incognitus*. For *B. sylvicola*, all of the

samples from Quail Mountain (the furthermost southwestern site) cluster together, but do not separate from the rest on the tree. Principal component analysis further reflected the extremely low level of geographical structure across all species (Fig. 2). Outlier clusters seen in the PCA plots of *B. balteatus* and *B. incognitus* (Fig. 2A,C, circled) are composed solely of the groups of sibling nestmates identified by the kinship analysis, suggesting that the effect of kinship overrides any signal of genetic structure. One slight exception to this trend is the Quail Mountain population of *B. sylvicola* which, as with the neighbor-joining tree, shows a slight degree of clustering away from the other samples at the positive extreme of PC1 (Fig.2B), but they still overlap with other samples and PC1 explains only 1.17% of the variance in the data.

We assessed connectivity between colonies on different mountains by analyzing the association between kinship and geography using a Mantel test. Mantel correlograms indicate a significant association between kinship and geographical distance at shorter distances (from the same mountain), which is no longer significant at larger distances (Fig. 3). Hence there is a no significant signal of increased degrees of kinship on neighboring mountains in our data.

Decrease in tongue length is ongoing in both species

We made morphological measurements for each sample (Table S2). Intertegular distance (ID) is considered a proxy for body size (Cane, 1987). We also measured the length of two parts of the tongue: glossa and prementum. These two tongue measures are correlated in both species (*B. balteatus* Pearson's $r = 0.45$, $p < 0.001$; *B. sylvicola* Pearson's $r = 0.38$, $p < 0.001$; Figs. S1,S2). Only glossa is considered further, as it is the tongue part with most relevance to feeding (Cariveau et al., 2016) and was measured by (Miller-Struttmann et al., 2015). Glossa is also significantly correlated with ID (*B. balteatus* Pearson's $r = 0.41$, $p < 0.001$; *B. sylvicola* Pearson's $r = 0.26$, $p < 0.001$) (Figs. S1,2).

A comparison of phenotypes between species shows that glossa length is substantially longer in *B. balteatus*, consistent with previous knowledge (ls-means = 4.72 mm, 3.34 mm, and 3.40 mm for *B. balteatus*, *B. sylvicola* and *B. incognitus* respectively; Tukey test, t ratio = 12.25 and 21.17, $p < 0.001$ for *B. balteatus* compared to *B. sylvicola* and *B. incognitus;*

414 Fig.4A). Average glossa length is extremely similar in *B. sylvicola* and B. *incognitus* (Tukey
415 test, t ratio =0.50, p = 0.87), suggesting that this character does not distinguish this newly-
416 discovered species from *B. sylvicola*. *Bombus balteatus* bees are significantly larger bees as
417 indicated by a greater ID, and *B. sylvicola* is significantly larger than *B. incognitus* (mean ID =
418 4.00 mm, 3.83 mm, 3.58 mm for *B. balteatus*, *B. sylvicola* and *B. incognitus* respectively;
419 ANOVA, F=28.75, p < 0.001, Tukey post-hoc test revealed differences are significant among
420 all species at p < 0.001; Fig. S3A).
421
422 We next compared the distribution of glossa lengths in our samples with historical data. We
423 combined *B. sylvicola* and *B. incognitus* for this analysis because they do not differ in glossa
424 length on average, and in order to compare to historical data in which the two species were
425 not distinguished. We find an overall trend of decreasing glossa length in both *B. sylvicola-*
426 *incognitus* and *B. balteatus* over time (Fig. 4B,C). This was reported previously between the
427 collections from 1966-1980 and from 2012-2014 for both species (Miller-Struttmann et al.,
428 2015). Here we show that this trend is continuing, with further reductions in glossa length
429 between the 2012-2014 collections and our collections from 2017. This reduction is
430 significant for *B. sylvicola-incognitus* (ls-means: 2012-2014 = 3.72 mm, 2017 = 3.24 mm;
431 Tukey test, t-ratio = 3.785, p < 0.001) and suggestive for *B. balteatus* (ls-means: 2012-2014 =
432 5.45 mm, 2017 = 5.11 mm; Tukey test, t-ratio = 2.026, p = 0.11). In contrast, there is no
433 significant trend shown by ID (Fig. S3B,C), although for *B. sylvicola-incognitus,* ID is
434 significantly larger in both the 1966-80 and 2017 samples compared to the 2012-2014
435 samples (ANOVA, F = 15.28, p < 0.001. Tukey post-hoc test, p < 0.001).
436
437 The trend for a decrease in glossa length occurs independently on all mountains for which
438 data is available in both species, but the decrease between 2012-2014 samples and 2017
439 samples is only significant for *B. sylvicola-incognitus* on Pennsylvania mountain (ls-means:
440 2012-2014 = 3.58 mm, 2017 = 2.96 mm, Tukey test, t-ratio = 3.61, p = 0.002 ; Fig. 4D,E).
441 There are no discernable historical trends over the same period for ID, and fluctuations in
442 opposite directions are observed among mountains (Fig. S3D,E).
443
444 For samples collected in 2017, we compared the distribution of glossa lengths for each
445 species among the seven mountains where they were collected (Fig. S4). There is an overall

446 trend for glossa lengths to be longer in bees from Mount Evans than on other mountains in
447 all species. The distributions for *B. balteatus* and *B. sylvicola* show significant differences,
448 with samples from Mount Evans having significantly longer glossae than on Mount
449 Democrat, Horseshoe Mountain, and Pennsylvania Mountain in *B. balteatus* (ls-mean glossa
450 length on Mt. Evans = 5.34 mm, other mountains 4.51 – 4.73 mm; Tukey $p < 0.01$ in each
451 case; Fig. S4A) and significantly longer glossae than on all other mountains except Niwot
452 Ridge in *B. sylvicola* (ls-mean glossa length on Mt. Evans = 3.77 mm, other mountains 3.02 –
453 3.24 mm; Tukey $p < 0.01$ in each case; Fig. S4B). None of the trends for *B. incognitus* were
454 significant (Fig. S4C). Comparisons of ID between mountains revealed that *B. balteatus* bees
455 sampled on Niwot Ridge are significantly larger than those on Boreas Mountain, Mount
456 Democrat, Horseshoe Mountain, and Quail Mountain (Niwot Ridge mean ID = 4.25 mm,
457 other mountains 3.84 - 4.00 mm, ANOVA, $F = 4.09$, $p < 0.001$, Tukey post-hoc test $p < 0.05$ in
458 four comparisons; Fig. S5A). We did not identify any significant differences in ID of bees
459 across mountains for *B. sylvicola* (ANOVA, $F = 1.83$, $p = 0.10$) or *B. incognitus* (ANOVA, $F =$
460 1.62, $p = 0.17$; Fig. S5B,C).
461
462 **Genome-wide association study identifies a SNP associated with a proxy for body size**
463 We used a linear mixed-model analysis to search for correlations between allele frequencies
464 and quantitative trait variation in *B. sylvicola* and *B. balteatus.* We used the traits glossa
465 length, ID and glossa length corrected for ID (see Methods). One from each pair of sisters
466 identified in the kinship analysis was removed from all analyses to reduce the level of
467 relatedness in the sample set. We detected a genome-wide significant association between
468 ID and a SNP in an intron of the *CTRB1* (Chymotrypsinogen B1) gene on chromosome 10 in
469 *B. sylvicola* ($p < 0.001$ after Bonferroni correction; Figs. 5, S6F). This gene is involved in
470 production of chymotrypsin, a key proteolytic digestive enzyme in bees, involved in
471 digestion of pollen. It is highly plausible that variation in this gene affects the rate of growth
472 and development in bees through its role in digestion. Two further SNPs, located 4 bp apart
473 on chromosome 11, also significantly associated with ID ($p = 0.039$ after Bonferroni
474 correction). However, they are not located in or close to any genes according to our
475 annotation, so any potential functional significance of these SNPs is unknown.
476

477 We did not find any significant associations between allele frequencies and ID in *B.*
478 *balteatus* (Fig. S6). Furthermore, no significant associations were found between allele
479 frequencies and glossa length, or glossa length corrected for ID in either species (Fig. S6).
480 This indicates that the trait has a polygenic or mainly environmental component. A power
481 analysis (Visscher et al., 2014) revealed that our datasets have sufficient power (>0.8) to
482 detect a QTL with a h^2 of ~0.1 but that loci with lower effect sizes would likely be
483 undetectable.
484
485 **Limited evidence that morphological variation has a genetic component**
486 We performed GCTA-GREML analyses (Yang et al., 2011) to determine the genetic
487 component of trait variation for *B. sylvicola* and *B. balteatus* (Fig. 6, table S4). The *B.*
488 *incognitus* samples were not included here due to smaller sample size. When each species'
489 entire dataset is considered, we detect a heritable component for both glossa length and ID
490 in both species, where the 95% confidence interval does not overlap zero. For *B. sylvicola*,
491 but not *B. balteatus*, this is also the case when ID is introduced as a covariate to account for
492 body size when considering glossa length. However, when we remove samples that we infer
493 to be workers from the same colony from the kinship analysis, we only detect a heritable
494 component with 95% confidence for ID in *B. balteatus* (all others then overlap with zero).
495 This indicates that a large proportion of the signal of covariance between genetic and trait
496 variance is due to the presence of sisters from the same colony. This could be a confounding
497 factor because environmental effects such as level of nutrition and colony health are likely
498 to covary with colony. However, such covariation could also reflect genetics. The level of
499 dependency of phenotype on genotype is therefore unclear from this analysis.
500
501 In these analyses, 95% confidence intervals are calculated by multiplying the standard error
502 by 1.96. As such, to achieve 95% confidence intervals that do not span the entire range of
503 possible heritability (0-1), standard errors must be kept low by increasing sample size. For
504 unrelated individuals it has been estimated that > 3,000 samples are required to achieve a
505 SE below 0.1 (Visscher et al., 2014).With our datasets, we have a power of ~0.06, or a ~6%
506 probability, to detect $h^2 > 0$ for a trait with a SNP-heritability of 0.5 and a type 1 error rate
507 (α) of 0.05. The low power of our datasets has therefore strongly influenced the estimates
508 we achieved.

509

510 **DISCUSSION**

511 In this study, we generated high-quality reference genomes for two bumblebee species: *B.*
512 *sylvicola* and *B. balteatus*. We also produced the most extensive population genomics
513 dataset for bumblebees to date, with 583 samples from these two species and the newly-
514 discovered *B. incognitus* collected from high-altitude locations in Colorado, USA. These data
515 were combined with morphometric measurements from each sample and used to
516 investigate genetic structure, morphological evolution and the genetic basis of phenotypic
517 traits in these populations. Our main findings are 1) no evidence for gene flow between but
518 lack of population structure within species, indicating high levels of connectivity among
519 subpopulations. 2) An ongoing decrease in tongue (glossa) length compared to historical
520 measurements, which likely reflects the continuing effects of decreasing floral abundance
521 under climate change on bumble bee foraging (Miller-Struttmann et al., 2015). 3) An
522 association between body size and variation at a single SNP located in a gene related to
523 production of a digestive enzyme in *B. sylvicola*. 4) A lack of conclusive evidence for a
524 genetic component to tongue length in both species. Our analysis is unable to determine
525 the degree of heritability underlying these traits but is consistent with a highly polygenic
526 genetic component.

527

528 This investigation adds to the number of bumblebee species with reference genomes
529 available. The genomes of the common species *B. impatiens* and *B. terrestris* were
530 sequenced using short-read technologies (Sadd et al., 2015). The availability of genetic maps
531 for *B. terrestris* allowed placement of contigs on chromosomes. Genome assemblies are also
532 available for representatives of all 15 *Bombus* subgenera (Sun et al., 2020) based on short-
533 read sequencing of jumping libraries and scaffolding using Hi-C, resulting in contig N50 of
534 325 kb. In addition to the two genomes produced here, the genomes of the North American
535 species *B. terricola*, *B. bifarius*, *B. vancouverensis*, and *B. vosnesenskii* have also been
536 produced using long-read sequencing (PacBio or ONT) and have megabase-scale contig N50s
537 (Heraghty et al., 2020; Kent et al., 2018).

538

539 Identification of the previously-unknown species *B. incognitus* demonstrates the power of
540 population-scale genome sequencing to discover new cryptic variation (Christmas,

541 submitted). None of the morphological features that we studied could distinguish between
542 *B. sylvicola* and *B. incognitus*. There were no significant differences in glossa or prementum
543 length. *Bombus sylvicola* did however have a significant tendency to be collected at higher
544 altitudes and have larger body size. The wider distribution of *B. incognitus* is unknown, as is
545 the true composition of populations described as *B. sylvicola* in other geographical
546 locations.
547
548 Another cryptic species, *B. interacti*, has been reported in Alaska, which is related to *B.*
549 *sylvicola* from North America and *B. lapponicus* from Eurasia (Martinet et al., 2019). This
550 species has subsequently been inferred to be synonymous with a previously-described
551 species found in the far north of North America, *B. johanseni* (Sheffield, Oram, & Heron,
552 2020). However, this species clusters more closely with *B. monticola* based on genetic data,
553 whereas *B. incognitus* displays closer affinity to *B. sylvicola* and *B. lapponicus* (Christmas,
554 submitted; Martinet et al., 2019). The same study also classified *B. sylvicola* as the same
555 species as *B. lapponicus*, which is found in Eurasia, due to a short genetic distance between
556 them (Martinet et al., 2019). It is however clear that the North American *B. sylvicola* and the
557 Eurasian *B. lapponicus* have experienced a prolonged period of geographic isolation, during
558 which intrinsic barriers to gene flow may have emerged. Further population genomic studies
559 of Pryobombus bumblebees from other geographic regions could potentially uncover new
560 cryptic species and determine species relationships and population structure at larger scale.
561
562 We find no evidence for geographical barriers to gene flow between subpopulations of any
563 of the three species studied here. Furthermore, although we observe elevated degrees of
564 relatedness among foragers collected on the same mountain, there is no significant
565 tendency for elevated relatedness of foragers caught on nearby mountains. These results
566 indicate that populations of these species are effectively panmictic across the area
567 surveyed, which has significance for their management. Foragers inferred to belong to the
568 same nest (i.e. with identical parents) were always found in the same or adjacent sites,
569 consistent with the short foraging distance (on average < 110m) inferred by (Geib et al.,
570 2015) for these species. Previous studies have used microsatellites to determine genetic
571 relatedness between individuals in order to survey colony density, foraging distance (Geib et
572 al., 2015), and lineage survival (Carvell et al., 2017). Whole genome sequencing is now a

573 relatively inexpensive alternative, which, together with availability of a reference sequence
574 assembly, can provide an accurate measurement of kinship between samples.
575
576 Morphological change over time has already been documented in the species studied here,
577 with a significant reduction in tongue length observed since 1966 (Miller-Struttmann et al.,
578 2015). During this time period, there has also been a shift in species composition, with
579 shorter-tongued species becoming more predominant. Previous collections of *B. sylvicola* in
580 this region are likely a mixture of *B. sylvicola* and *B. incognitus*, as the existence of *B.*
581 *incognitus* was unknown. However, as *B. sylvicola* and *B. incognitus* do not differ in glossa
582 length, undetected changes in the relative abundance of these species over time are
583 unlikely to be responsible for the observed shifts in glossa length observed in samples that
584 are defined as *B. sylvicola.* This effect could however contribute to the observed variation in
585 body size over time as *B. sylvicola* have a significantly greater ID on average than *B.*
586 *incognitus* (Figure S3).
587
588 A major finding that we present here is that the temporal shift toward shorter tongue
589 length is ongoing. In addition to the significant reduction previously observed over a period
590 of six decades up until 2014, we also observe a significant reduction over a much shorter
591 time period between 2012-14 and 2017 in *B.sylvicola-incognitus*. Our results also indicate
592 that the shift has not only continued, but has accelerated compared to previous
593 measurements. Mean tongue length in *B. sylvicola* decreased on average by 0.61% annually
594 in a period between 1966 and 2014. According to our recent measurements, the
595 corresponding decrease in 3-5 subsequent years has been 12.9 %, or 3.2% per year
596 (compared to the 2012-14 average). These changes have been previously attributed to a
597 connection between shorter tongues and generalist foraging, a strategy which is favored at
598 lower floral density. It is suggested that these conditions have become more common in the
599 habitats of these species in the study area due to the effects of climate change (Miller-
600 Struttmann et al., 2015). We also observe significant differences in tongue length between
601 mountains, which could potentially reflect variation in these environmental factors between
602 mountains. In contrast to tongue length, ID has remained relatively constant despite some
603 fluctuations.
604

605 Our results are consistent with a hypothesis that tongue length changes are driven by
606 natural selection due to changes in flower abundance driven by climate change. There are
607 now many examples of rapid morphological evolution driven by natural selection related to
608 climatic events in the wild, such as a shift in beak shape over a period of a few years in
609 Darwin's finches related to competition for food during drought (Lamichhaney et al., 2016)
610 and genomic shifts related to cold-tolerance in the green anole lizard related to severe
611 winter storms (Campbell-Staton et al., 2017). Human activity may also promote rapid
612 adaptation by natural selection, as demonstrated by the evolution of longer beaks in great
613 tits promoted by the use of bird feeders in the UK (Bosse et al., 2017).
614
615 It is however unclear whether the observed changes in tongue length due to natural
616 selection acting on genetic variation. It is also possible that the changes in tongue length we
617 observe are due to plasticity. In order to address this question, we attempted to uncover
618 the genetic basis of morphological variation using genome-wide association studies (GWAS)
619 and genome-wide complex trait analysis (GCTA). No significant associations were observed
620 for tongue length in the genome of any of the species. However, a significant association
621 with body size was identified in *B. sylvicola* in the *CTRB1* (Chymotrypsinogen B1) gene. This
622 gene produces of a precursor of chymotrypsin, which is involved in proteolysis and likely
623 plays a role in digestion of pollen (Grogan & Hunt, 1979; Lazarević & Janković-Tomanić,
624 2015; Rawlings & Barrett, 1994). It is therefore highly plausible that variation in the activity
625 of this enzyme could influence body size, and intriguing that a variant of relatively large
626 effect is segregating in this natural population.
627
628 We detected a significant signal of covariance of trait measurements with relatedness using
629 GCTA when closely-related individuals were included in the dataset. However, no significant
630 signal of heritability in morphological traits was detected when the dataset was purged of
631 close relatives. The first result could potentially reflect a genetic component, but it is not
632 possible to disentangle this component from the shared environmental effects due to close
633 relatives sharing a nest. This analysis is therefore underpowered to detect genetic effects
634 due to low sample size and a general lack of related individuals in the dataset. We estimated
635 that a sample size 10-times larger would be necessary to estimate heritability with a
636 standard error below 0.1 (Visscher et al., 2014). For polygenic traits, such as body size and

637 tongue length, there are likely multiple QTL of small effect ($h^2 < 0.1$) throughout the genome
638 (Yang et al., 2010) and it is therefore not surprising that such loci were not detected. A study
639 of such a magnitude in a wild bumblebee population would require a huge sampling effort
640 that could have detrimental efforts on colony survival. A probable explanation for our
641 results is that the morphological traits have a highly polygenic genetic component, and
642 GWAS and GCTA were underpowered to detect QTL and estimate heritability. The size of
643 the genetic component of variation in both traits in each species is therefore still unknown.
644
645 In summary, we have shown here that morphological change under climate change is still
646 ongoing in alpine bumblebee populations at high-altitudes in Colorado. We generated the
647 genomic resources to study these species on the genetic level in the form of highly-
648 contiguous genome assemblies and comprehensively characterized genome variation. Our
649 results identified a previously-undescribed species and provided insights into population
650 structure and the genetic basis of morphological variation in these populations. Alpine
651 ecosystems are particularly sensitive to climate change and may suffer its effects before
652 other regions (Elsen & Tingley, 2015). Continued monitoring of changes in morphology and
653 species abundance of the populations under study here would be highly valuable to
654 understand how species elsewhere will adapt in future. Similar genomic studies of these
655 bumblebee subgenera in other mountain and artic locations would also be valuable in order
656 to fully characterize species diversity, including additional cryptic species, and to further
657 understand the ecological and genetic causes for morphological variation.
658
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DATA ACCESSIBILTY

All sequence data is associated with NCBI project IDs: PRJNA646847 and PRJNA704506

AUTHOR CONTRIBUTIONS

Designed research: MJC, NEM-S, JCG, MTW; Performed research: all authors; Analyzed data: MJC, JCJ, OW, IB, MK, MTW; Wrote the paper: MJC, MTW.

Table 1: Assembly metrics for the two genomes, with two other published *Bombus* genomes included for comparison

Species	*B. balteatus*	*B. sylvicola*	*B. terrestris*	*B. impatiens*
Assembly	BBAL_1.0	BSYL_1.0	Bter_1.0	BIMP_2.2
Size (Mbp)†	250.07	252.08	248.65 (236.38)	246.86 (241.98)
Scaffolds (n)	-	-	5,678	5,460
Contigs (n)	336	592	10,672	16,060
Contig N50 (Mbp)	8.60	3.02	0.08	0.06
Contig L50 (n)	12	28	890	54
GC (%)	37.64	38.23	37.51	37.76
Repeat content (%)	17.1	17.9	14.8	17.9
Complete hymenoptera BUSCO genes (%)*	99.0	98.2	96.9	98.3

*BUSCO analysis using the OrthoDB v. 10, Hymenoptera dataset.

† Numbers in brackets represent total ungapped length

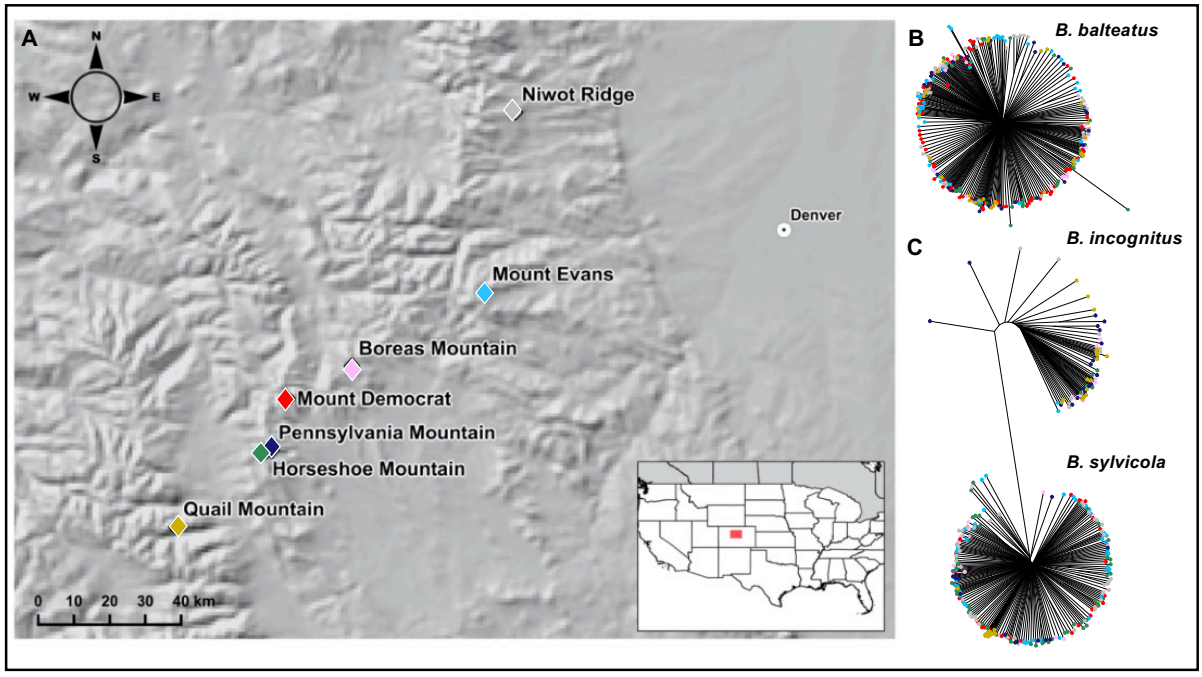

Figure 1. Population sampling and genetic variation in three *Bombus* species. (A) Map showing the sampling locations of *Bombus balteatus, B. sylvicola,* and *B. incognitus* on seven mountains in the Rocky Mountains, CO, USA. Insert indicates location in the USA. Neighbour-joining trees of (B) *B. balteatus* and (C) *B. sylvicola* and *B. incognitus* populations sampled across the seven mountains, based on genome-wide single nucleotide polymorphisms. Coloured tips indicate the mountain each sample was collected on, with colours corresponding to those in (A).

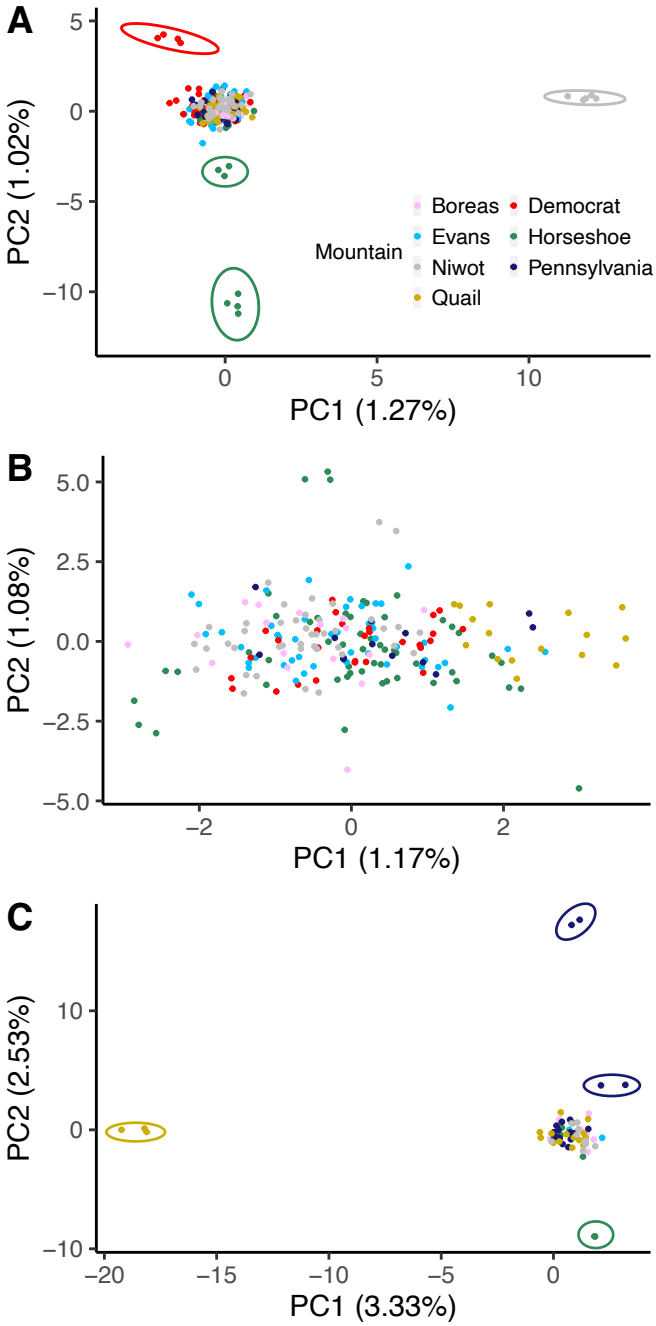

Figure 2. Principal component analysis of Rocky Mountain populations of three *Bombus* species. Plots show the first two principal components from principal component analyses of (A) *Bombus balteatus*, (B) *B. sylvicola* and (C) *B. incognitus* populations. Numbers in brackets on axes indicate the percentage variance explained by each principal component. Samples in clusters indicated by ellipses in (A) and (C) are full sisters.

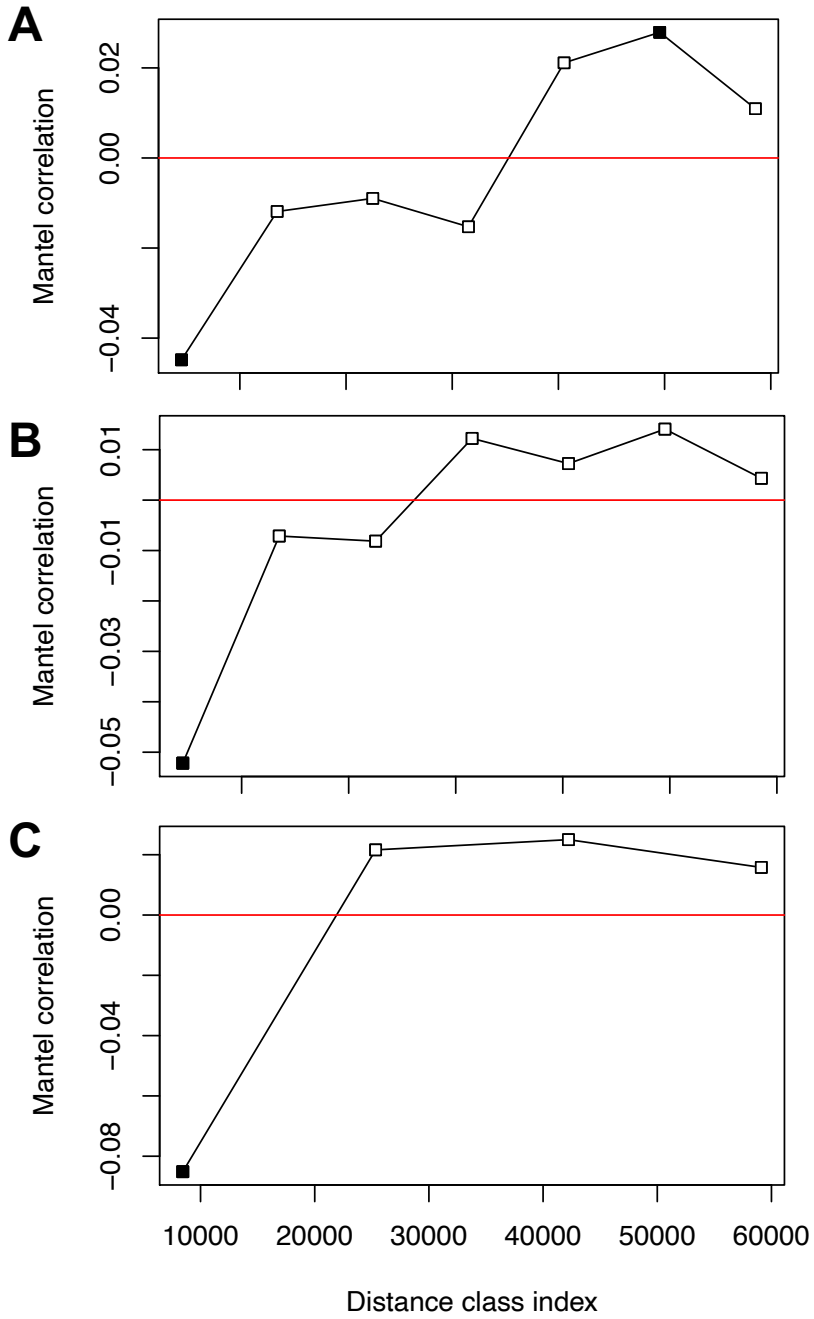

Figure 3. Mantel correlograms showing correlations between kinship and geographic distance in different distance classes. The Mantel correlation between a kinship matrix and a geographic distance matrix (y-axis) is calculated over 15 distance classes (x-axis, in metres) for (A) *Bombus balteatus,* (B) *B. sylvicola*, and (C) *B. incognitus*. Filled in boxes indicate significant correlations ($p < 0.01$). In all three cases, there is a significant negative correlation in the first distance class (< 10 km), indicating the shorter the distance the higher the relatedness over short distances. This reflects the fact that nest mates forage over short distances. At greater distance classes, the correlations are mostly nonsignificant (open boxes). Distances over 60 km had insufficient data points for calculating Mantel correlations.

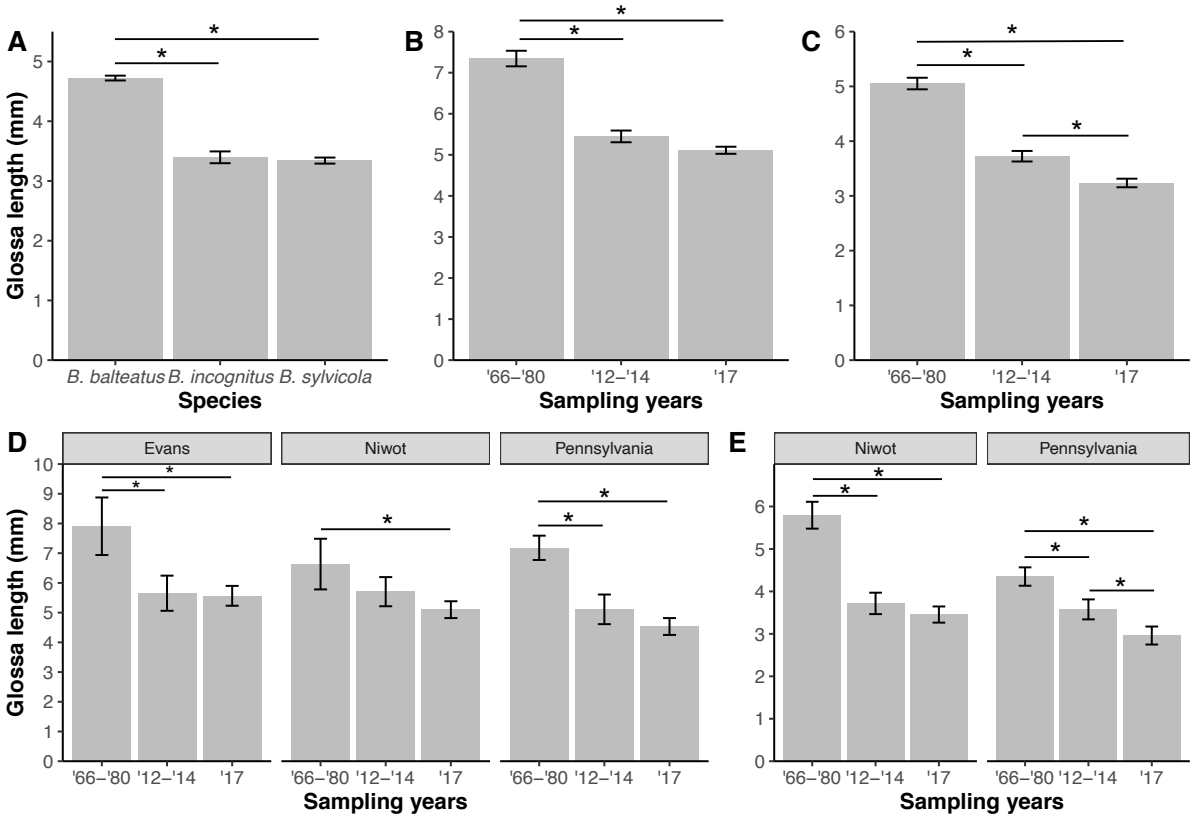

Figure 4. Comparisons of glossa lengths between species, years, and locations. (A) Least-squares mean (ls-mean) glossa lengths correcting for body size for the three *Bombus* species, based on measurements of the 2017 collections. Least-squares mean glossa lengths per sampling period for (B) *B. balteatus* and (C) *B. sylvicola* and *B. incognitus* combined based on measurements made from samples collected at the same locations in each time period. These locations are shown in (D) for *B. balteatus* and (E) for *B. sylvicola-incognitus*, where the samples have been separated out per location. In all plots, error bars represent ls-means ± standard error and significant differences between classes are indicated by asterisks ($p < 0.05$).

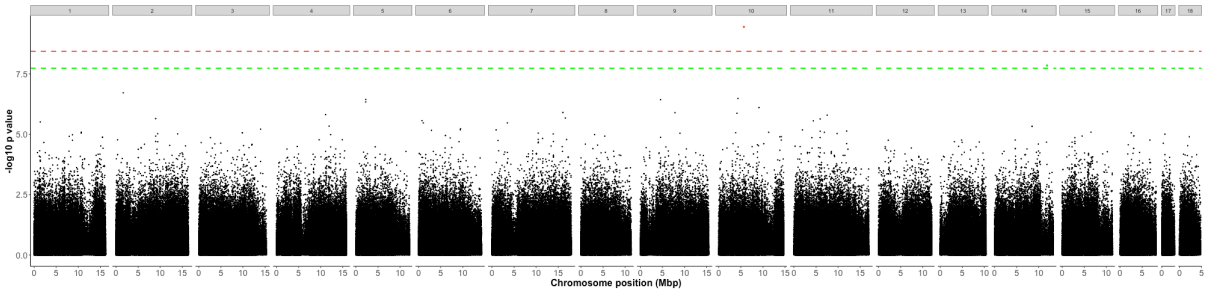

Figure 5. Genome-wide association Manhattan plot. The -$\log_{10}$ p-values from an association test using a mixed linear model between allele frequencies and intertegular distance (ID) are plotted for 2,705,361 genome-wide SNPs against their genome positions over the 18 *Bombus* chromosomes. Red dashed line indicates genome-wide significance after Bonferroni correction for p = 0.01. A single SNP on chromosome 10, coloured red, had a -$\log_{10}$ p-value above this threshold. Green dashed line indicates genome-wide significance after Bonferroni correction for p = 0.05. Two neighbouring SNPs located on chromosome 14, coloured green, had -$\log_{10}$ p-values above this threshold.

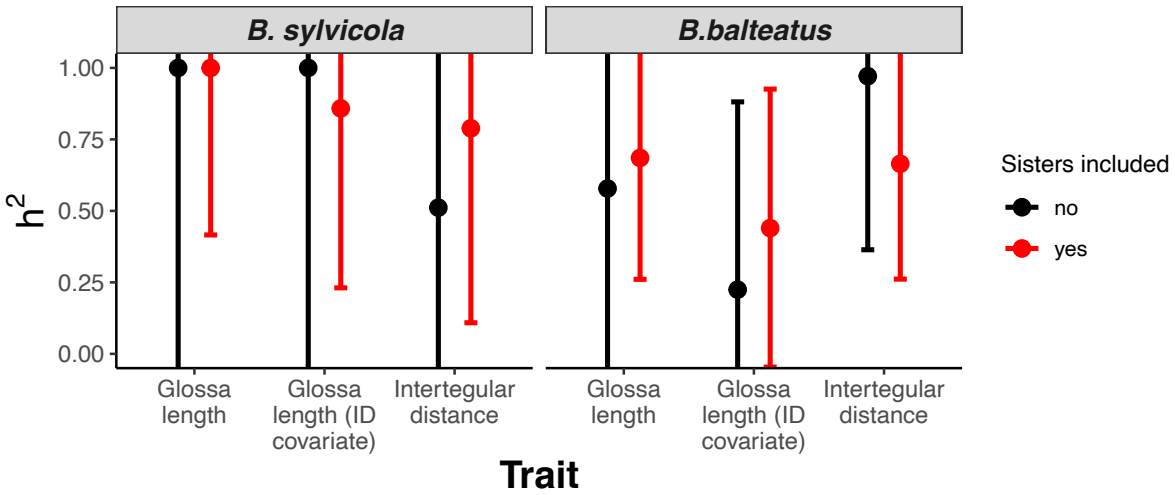

Figure 6. Estimates of SNP-heritability for two traits in *Bombus* species. The proportion of variance in glossa length and intertegular distance (ID) explained by all genome-wide SNPs (the SNP-based heritability) was calculated using the genome-base restricted maximum likelihood (GREML) method in GCTA. These were calculated separately for *B. sylvicola* (left panel) and *B. balteatus* (right panel). We included ID as a covariate with glossa length to control for body size. Error bars indicate 95% confidence intervals around estimates. Each analysis was carried out twice, once with all samples (red), and a second time with one from each pair of sisters removed (black), in order to assess the effect of including highly related samples in the analysis.