A genomic and morphometric analysis of alpine bumblebees: ongoing reductions in tongue length but no clear genetic component

Running title: Genome variation in alpine bumblebees

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ABSTRACT

Over the last six decades, populations of the bumblebees *Bombus sylvicola* and *Bombus balteatus* in Colorado have experienced decreases in tongue length, a trait important for plant-pollinator mutualisms. It has been hypothesized that this observation reflects selection resulting from shifts in floral composition under climate change. Here we used morphometrics and population genomics to determine whether morphological change is ongoing, investigate the genetic basis of morphological variation, and analyze population structure in these populations. We generated a genome assembly of *B. balteatus*. We then analyzed whole-genome sequencing data and morphometric measurements of 580 samples of both species from seven high-altitude localities. Out of 281 samples originally identified as *B. sylvicola*, 67 formed a separate genetic cluster comprising a newly-discovered cryptic species ("incognitus"). However, an absence of genetic structure within species suggests that gene flow is common between mountains. We found a significant decrease in tongue length between bees collected between 2012-2014 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 was implicated in body size variation. We identified 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 possible that shared environmental effects between colonies are responsible. Our results provide evidence for ongoing modification of a morphological trait important for pollination and indicate that this trait likely has a complex genetic and environmental basis.

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, 2008a; 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, and can be considered "canaries in the coal mine" for detecting early effects (Elsen & Tingley, 2015).

In addition to range shifts there is evidence that the morphology of certain populations of bumblebees has shifted in recent decades. Miller-Struttmann et al. (2015) compared morphometric measurements of contemporary samples and historical specimens of the species *B. balteatus* and *B. sylvicola* from the same high-elevation alpine localities in Colorado. These species historically comprise the majority of bumblebees in these locations (Miller-Struttmann et al., 2015). 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 drier soils resulting from climate change. These conditions could favor more generalist foraging strategies, which several studies indicate are performed more efficiently by bees with shorter tongues (Goulson & Darvill, 2004; Goulson, Hanley, Darvill, Ellis, & Knight, 2005; Goulson, Lye, & Darvill, 2008b; Heinrich, 2004; Huang, An, Wu, & Williams, 2015). Continued monitoring of these populations is important to understand the causes of these morphological shifts and their ecological consequences. It is unknown whether the trend towards 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 also possible that the observed changes are a plastic response to environmental change (Merilä & Hendry, 2014). It is unknown whether this trait has a 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 any genetic loci involved would be an important step towards understanding the underlying cause of the morphological changes. The feasibility of genome sequencing on a population scale has enabled studies of quantitative genetics (Gienapp et al., 2017) and genome-wide association studies (Santure & Garant, 2018) in wild populations, allowing these goals to be pursued.

The genetic structure of populations is an important factor for interpretation of genotype-phenotype correlations and the cause of morphological shifts. We previously presented a

genome assembly of *B. sylvicola* and analyzed genome-wide variation by resequencing 281 samples (Christmas et al., 2021). This identified the presence of a previously-undetected cryptic species, which we gave the preliminary name *Bombus incognitus*. We refer to this species hereafter simply as "incognitus" to reflect the fact that a formal taxonomic description is not currently available. This species was identified morphologically as *B. sylvicola* but forms a distinct genetic cluster. It is therefore possible that changes in tongue length 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 genetic structure and 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, Nowak, Bálint, & Pfenninger, 2013; Razgour et al., 2019).

The degree of gene flow in bumblebee populations is determined by the distances that reproductive individuals (males and queens) travel in order to mate and establish new nests (Heinrich, 2004; Woodard et al., 2015). Genetic methods have been used to investigate population structure of several species of bumblebees across a variety of habitats in the UK, continental Europe and continental USA (Darvill, Ellis, Lye, & Goulson, 2006; Ellis, Knight, Darvill, & Goulson, 2006; Ghisbain et al., 2020; Jackson et al., 2018; Jha, 2015; Koch, Looney, Sheppard, & Strange, 2017; Woodard et al., 2015). A general finding is that populations of common species tend to exhibit very little structure in the absence of geographical barriers, even at continental scales. However, genetic differentiation can occur in rare or declining species, or when there are natural barriers (Woodard et al., 2015). Populations restricted to high elevations have been shown to exhibit higher genetic differentiation (Lozier, Strange, & Koch, 2013; Lozier, Strange, Stewart, & Cameron, 2011). However, the degree of fragmentation of populations of bumblebee species inhabiting high elevations in mountain ranges is not fully understood.

Genetic methods can also be applied to estimate nest density and foraging distances of bumblebees, by analyzing where genetically-related nestmates are caught. Such studies indicate that maximum foraging distances range from less than 100 m to over 10 km

124 (Darvill, Knight, & Goulson, 2004; Geib, Strange, & Galen, 2015; Jha & Kremen, 2013; Knight
125 et al., 2005; Woodard et al., 2015). These variables show large variation among species and
126 are strongly dependent on landscape composition. Genome sequencing of multiple
127 individuals has the potential to add greater resolution to our understanding of population
128 density, structure and dispersal.
129
130 In this study we present a high-quality annotated genome assembly of the bumblebee
131 species *B. balteatus* based on long-read Oxford Nanopore sequencing. We also conducted
132 whole-genome sequencing of 299 samples of this species sampled from seven high-altitude
133 localities spread across 150 km in Colorado. These localities have an average separation of
134 54 km between each other, and consist of alpine tundra separated by forest. We analyze
135 these data together with the previously-published population genomic dataset of 284
136 samples of *B. sylvicola* and "incognitus" from the same localities (Christmas et al., 2021). We
137 use these data to assess whole-genome variation and its connection to geographical and
138 morphological variation in these populations, which were previously studied by Miller-
139 Struttmann et al. (2015). We then analyzed morphological variation in tongue length and
140 body size in context of previous studies (Miller-Struttmann et al., 2015) to determine
141 whether the trend towards decreasing tongue length is ongoing. We also performed a
142 genome-wide analysis of the genetic basis of morphological variation using both genome-
143 wide association studies (GWAS) and genome-wide complex trait analysis (GCTA) (Yang, Lee,
144 Goddard, & Visscher, 2011). The results are informative regarding the degree of connectivity
145 among bumblebee populations, the genetic basis of morphological variation, and the
146 mechanisms of morphological evolution.
147
148 Our study species have recently been subject to taxonomic revision. The species we refer to
149 here as *B. balteatus* was previously described as occurring in both North America and
150 Eurasia, but a recent study classified these geographically separated populations as two
151 different species: *B. balteatus* in Eurasia and *Bombus kirbiellus* in North America (Williams et
152 al., 2019). The species we refer to here as *B. sylvicola* was previously split into a North
153 American form (*B. sylvicola*) and a Eurasian form (*Bombus lapponicus*). However, a recent
154 analysis synonymized these two species as *B. lapponicus* (Martinet et al., 2019). We

continue to use the previous names here to avoid confusion with comparison to previous ecological and evolutionary genetic studies.

MATERIALS AND METHODS

Sample collection

We collected samples identified as *B. balteatus* and *B. sylvicola* (Koch, Strange, & Williams, 2014) during the summer of 2017. Bumblebees were collected on seven different mountains in Colorado, USA, with an average distance of 54 km between mountains (greatest distance: 134 km, Quail Mountain – Niwot Ridge; shortest distance: 10km, Mount Democrat – Pennsylvania Mountain). The existence of "incognitus" was unknown to us when the bees were sampled but a large fraction of bees identified as *B. sylvicola* were subsequently assigned to "incognitus" on the basis of genetic analysis (Christmas et al., 2021). Three of these mountains (Mount Evans, Niwot Ridge, and Pennsylvania Mountain) were also sampled previously by Miller-Struttmann et al. (2015). Four additional mountains were sampled here (Boreas Mountain, Mount Democrat, Horseshoe Mountain and Quail Mountain) but were not sampled in the previous study. Each of the mountain locations comprised several neighboring sampling sites separated by short distances (mean = 1.1 km) and elevations (range: 3,473 – 4,012 m.a.s.l.) (full details in supplementary tables S1, S2).

We collected samples of foraging worker bees from each site using sweeping hand nets. We kept samples in Falcon tubes in cool boxes for transport. Species assignment was performed by observing standard morphological characters (Koch et al., 2014). We dissected each bee after placing it at -20°C for approximately 10 minutes.

Morphological measurements

We measured intertegular distance and tongue length on all of the samples of *B. sylvicola,* "incognitus" and *B. balteatus* using scaled photographs of individual bee heads processed by ImageJ (Kearns & Inouye, 1993; Schneider, Rasband, & Eliceiri, 2012). Following netting, bees were anesthetized by chilling them on ice or in a freezer (depending on field conditions). Once anesthetized, the bees were removed from the vial, gently pressed prone to the table with the thorax parallel to the camera lens using flat forceps, and photographed with a metric ruler for scaling purposes. The distance between the tegulae was measured as

187 a straight line between the edge of one tegula to the other tegula, running as near to
188 perpendicular to the tegula as possible. The head was then immediately removed from the
189 body and placed into an individual labeled vial and kept frozen until further characterization
190 in the lab. Following the protocol used in Miller-Struttmann *et al.* (2015), the prementum
191 and glossa were dissected from the head while the tissue was flexible, straightened and
192 aligned as possible without damaging the tissue, and mounted on acid-free paper. The
193 samples were then photographed with a ruler in the field of view under a microscope, and
194 the traits measured (i.e., prementum length and glossa length) via ImageJ (Schneider et al.,
195 2012). After photographing, we stored thoraces in 95% ethanol prior to DNA extraction. We
196 visually characterized the diagnostic traits visible on each head, such as the shape of the
197 malar space, the pile color between and antenna and above the ocelli, the size, color and
198 location of the ocelli relative to the supraorbital line, and the presence of black pile on the
199 scutellum. None of the morphological characters we measured could be used to distinguish
200 between "incognitus" and *B. sylvicola*.
201
202 **Genome sequencing and assembly**
203 We used the same sequencing strategy to produce high-quality genome assemblies for both
204 *B. sylvicola* and *B. balteatus*. The *B. sylvicola* assembly was presented previously (Christmas
205 et al., 2021). This involved combining data from Oxford Nanopore (ONT) and 10x Genomics
206 Chromium technologies. For both species, we used DNA extracted from male bees sampled
207 on Niwot Ridge and stored in 95% ethanol prior to DNA extraction. DNA from a single bee
208 was used for each assembly. We performed DNA extraction using a salt-isopropanol
209 extraction followed by size selection using magnetic bead purification to remove fragments
210 < 1 kbp. ONT sequencing was performed using the MinION instrument. For *B. sylvicola*, we
211 used two R9.4 flowcells and the RAD004 kit starting with 3-400 ng DNA per run. This
212 resulted in a total of 9.4 Gbp of sequence data, in 2.5 million reads with a mean length of
213 3.7 kbp (Christmas et al., 2021). For *B. balteatus*, we used 1µg DNA in a modified LSK-108
214 one-pot protocol. 1.75 µl NEB FFPE buffer, 1.75 µl NEB Ultra-II End Prep buffer and 1 µl FFPE
215 repair mix was added to 24 µl DNA and incubated at 20°C for 30 minutes. 2 µl End Prep
216 enzyme mix was then added for blunting, phosphorylation and A-tailing (20 min. 20°C, 10
217 min. 65°C, 10 min. 70°C). AMX (20 µl), ligation enhancer (1 µl) and Ultra-II ligase (40 µl) was
218 added for ligation at room temperature for 30 minutes. Clean-up and flowcell loading was

219 done following the standard ONT LSK-108 protocol and the library was sequenced on one
220 R9.4 flowcell. This resulted in 23.3 Gpb of sequence data, in 3.9 million reads with a mean
221 length of 5.9 kbp.
222
223 We assembled the ONT reads using the following process: We first used downpore
224 (Teutenberg, 2018/2020) with default parameters for adaptor trimming and splitting
225 chimeric reads. We then assembled the trimmed reads using wtdbg2 (Ruan & Li, 2020) on
226 default settings. We then ran two rounds of the consensus module Racon (Vaser, Sović,
227 Nagarajan, & Šikić, 2017) and contig improvements using medaka v.0.4 (*Medaka*,
228 2017/2021), removing contigs of length < 20 kbp. We used Illumina short-read data from
229 the population sequencing to perform two rounds of Pilon (Walker et al., 2014) to improve
230 sequence accuracy, particularly around indels.
231
232 We also sequenced the same sample of each species using 10x Genomics Chromium. A 10x
233 GEM library was constructed according to the manufacturer's protocols. We normalized
234 each library using qPCR and sequenced them together on a single lane of HiSeq 2500 using
235 the HiSeq Rapid SBS sequencing kit version 2, resulting in 150 bp paired-end sequences. The
236 linked reads were mapped to the assembly using Longranger v.2.1.4
237 (*10XGenomics/Longranger*, 2018/2021). We then ran Tigmint v1.1.2 (Jackman et al., 2018)
238 to correct errors in the assembly detected by the read mappings. We identified contigs that
239 contained mitochondrial genes, and were therefore likely fragments of the mitochondrial
240 genome, by running a BLAST search *of B. impatiens* mitochondrial genes across the
241 assembly using BLAST+ v2.9.0 (Camacho et al., 2009). Any contigs containing two or more
242 mitochondrial genes located within the expected distance of each other based on their
243 locations on the mitochondrial genome were removed from the assembly to ensure the
244 final assembly did not contain partially assembled mitochondrial genome sequence. We also
245 removed all contigs shorter than 10 kbp.
246
247 **Genome analysis**
248 BUSCO v3.0.2b (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015) was used to
249 assess the assembly completeness using the hymenoptera_odb9 lineage set and species *B.*
250 *impatiens*. We ran RepeatMasker v.4.1.0 (Smit, Hubley, & Green, 2015) on each genome

251 assembly to characterize genome repeat content, including interspersed repeats and low
252 complexity sequences. Chromosome-level assemblies of species from five diverse
253 bumblebee subgenera indicate that non-parasitic clades have a stable 18 chromosome
254 karyotype (Sun et al., 2020). We therefore performed whole-genome synteny alignments
255 between the *B. terrestris* chromosome-level genome assembly (Sadd et al., 2015)
256 (downloaded from NCBI; BioProject PRJNA45869) and our assemblies using Satsuma v.3
257 (Grabherr et al., 2010) to arrange the contigs from our assemblies onto 18
258 pseudochromosomes.
259
260 **RNAseq and Annotation**
261 We used the Nextflow pipelines available at https://github.com/NBISweden/pipelines-
262 nextflow for genome annotation. The pipelines, in turn, depend on two additional
263 annotation-specific toolkits, AGAT and GAAS, available
264 at https://github.com/NBISweden/AGAT and https://github.com/NBISweden/GAAS
265 respectively. Both repositories contain extensive documentation and installation
266 instructions. As reference proteins database, we used the SwissProt/UniProt (561,356
267 curated proteins, downloaded 2019-11). The parameter files stated below are available at
268 https://github.com/matt-webster-lab/alpine_bumblebees.
269
270 We performed RNAseq on a single lane of Illumina HiSeq 2500 using samples from four body
271 parts from a single sample of *B. balteatus* (abdomen, head, legs and thorax), which
272 generated 42 Gbp of reads. We performed quality control of the data using FastQC
273 (Andrews, 2017/2021) and read trimming using trimmomatic (v. 0.39,
274 trimmomatic.params). We next generated a guided assembly with Hisat2 (Kim, Paggi, Park,
275 Bennett, & Salzberg, 2019) and Stringtie (Pertea et al., 2015) using the
276 AnnotationPreprocessing.nf (parameters as in AnnotationPreprocessing_params.conf)
277 TranscriptAssembly.nf Nextflow pipeline with parameters as in the
278 TranscriptAssembly_params.config file. In addition to the guided assembly, we performed a
279 de novo assembly for reads from each of the four tissues using Trinity v. 2.0.4 (Grabherr et
280 al., 2011) (--seqType fq --SS_lib_type RF).
281

282 Next, we constructed a species-specific repeat library using RepeatModeler package (v.
283 open-1.0.8; -engine ncbi -pa 35). Since the de novo identified repeats may still include parts
284 of protein-coding genes, we perform an additional filtering step and remove from the
285 database the repeats that can be found in a comprehensive set of known proteins. We
286 remove all repeats that match to a pre-computed set non-transposable proteins from our
287 protein reference database. We used custom script (filter_repeats.bash) for filtering. To
288 identify the location of repeats from this library in the genomes, we used RepeatMasker
289 (open-4.0.9).
290
291 We used the MAKER (Cantarel et al., 2008) package to compute gene builds. An evidence-
292 guided gene build was computed by MAKER (-fix-nucleotides) constructing gene models
293 from these reference proteins and from the transcript sequences aligned to the genome
294 assembly (parameters in evidence_build.maker_opts.ctl). We also prepared an *ab initio*
295 build using the AbInitioTraining.nf pipeline and AbInitioTraining_params.config as the
296 parameters file. Both the evidence-guided and the ab initio builds were than merged using
297 MAKER (hybrid_build_maker_opts.ctl). Finally, we performed functional annotation using
298 the FunctionalAnnotation.nf pipeline with FunctionalAnnotation_params.config as the
299 parameter file.
300
301 **Population sequencing, read mapping and variant calling**
302 We extracted DNA from the thoraces of 299 worker bees of *B. balteatus* collected from
303 across the seven sampling sites using the Qiagen Blood and Tissue kit. We prepared dual-
304 indexed libraries using the Nextera Flex kit and performed sequencing on an Illumina HiSeq
305 X to produce 2 x 150 bp reads, using an average of 36 samples per lane. We mapped reads
306 to the *B. balteatus* reference using the mem algorithm in BWA (Li & Durbin, 2009). We
307 performed sorting and indexing of the resultant bam files using samtools (Li et al., 2009) and
308 marked duplicate reads using Picard. We used the Genome Analysis Toolkit (GATK) to call
309 variants (McKenna et al., 2010). We first ran HaplotypeCaller using default parameters on
310 the bam file of each sample to generate a gVCF file for each sample. We then used
311 GenomicsDBImport and GenotypeGVCFs with default parameters to call variants for all *B.*
312 *balteatus* samples together. We applied a set of hard filters using the VariantFiltration tool
313 to filter for reliable SNPs using the following thresholds: QD < 2, FS > 60, MQ < 40,

314 MQRankSum < -12.5, ReadPosRankSum < -8 (see the GATK documentation for full
315 descriptions of each filter). Only biallelic SNPs were considered for downstream analysis.
316 Samples from *B. sylvicola* and "incognitus" samples were analyzed in the same way and are
317 presented in Christmas *et al.* (2021).
318
319 **Relatedness and population structure**
320 We estimated the pairwise kinship coefficient between all samples within each species using
321 the method described in (Manichaikul et al., 2010) implemented in the --relatedness2
322 option of vcftools v0.1.16 (Danecek et al., 2011). Worker bees from the same bumblebee
323 colony are expected to have a relatedness of 0.75 under the assumption of monandry,
324 which is reasonable for the study species (Estoup, Scholl, Pouvreau, & Solignac, 1995). The
325 kinship coefficient (φ) predicted from this relationship is $\frac{3}{8}$ (the probability that two alleles
326 sampled at random from two individuals are identical by descent). We estimated the lower
327 cutoff for the inference criteria to infer this level of relatedness using powers of two as
328 described in (Manichaikul et al., 2010), providing an estimate of $\frac{3}{2^{7/2}}$.
329
330 We tested for correlations between relatedness and geographic distance for each species
331 using Mantel tests with the mantel function in the 'ecodist' package in R v4.0.2. Coordinates
332 for each sample were converted to geographic distances using the R package 'geodist' and
333 these were compared to the matrix of kinship coefficients described above. We used the
334 mantel.correlog function to produce mantel correlograms and assess whether correlations
335 were significant over different geographic distances. We performed these analyses after
336 excluding sisters from the datasets to ensure any patterns we observe were not driven by
337 an effect of sampling individuals from the same colony.
338
339 We thinned the VCF files to 1 SNP every 10 kbp using the --max-missing 1 and --thin flags in
340 vcftools v.0.1.16 to ensure no missing data and to assess population structure and
341 relatedness more efficiently. We calculated p-distance matrices for *B. balteatus* samples and
342 *B. sylvicola* and "incognitus" samples combined from the thinned vcf files using VCF2Dis
343 (https://github.com/BGI-shenzhen/VCF2Dis) and then constructed neighbor-joining trees
344 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 performed PCAs on all samples as well after removing one of each pair of sisters identified via the kinship analysis to ensure that relatedness did not heavily influence any clustering. 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.

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 intertegular distance, 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 intertegular distance 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.

Within our 2017 dataset, we compared glossa lengths between the three species by fitting linear models of species against glossa length with intertegular distance included as a random effect to account for body size using the lm function. We then calculated least-squares mean glossa lengths from this model using the lsmeans function and tested for significant differences using the Tukey method for p-value adjustment for multiple testing. We employed a similar approach for comparing glossa lengths between our dataset and historical datasets for each species. Here we used a linear mixed effect model framework with the 'lmer' function in the lme4 package in R v4.0.2, where mountain and intertegular distance were included as random effects in each model. Significance of each model was assessed using analysis of variance with the 'anova' function. We calculated least-squares mean glossa lengths from each model using the lsmeans function and tested for significant differences using the Tukey method for p-value adjustment for multiple testing.

377 We analyzed variation in intertegular distance between species and geographic locations for
378 our 2017 dataset using analysis of variance ('aov' function) and assessed significance of
379 comparisons using Tukey's honestly significant difference (HSD) post hoc test ('TukeyHSD'
380 function). For comparisons of intertegular distance between years, we fitted linear mixed
381 effect models and included mountain as a random effect, then compared least-squares
382 means from the models using the Tukey method for p-value adjustment for multiple testing.
383

384 **Genome-wide association study**

385 We carried out mixed linear model-based association analyses, implemented in GCTA-
386 MLMA (Yang et al., 2011; Yang, Zaitlen, Goddard, Visscher, & Price, 2014), to identify
387 correlations between allele frequencies and trait variation across the genome for *B.*
388 *balteatus* and *B. sylvicola* samples separately ("incognitus" samples were left out of this
389 analysis due to too few samples). First, we created a genetic relationship matrix (GRM) from
390 the genome-wide SNPs using the --make-grm function in GCTA. We removed one sample
391 from each pair of sisters by setting a relatedness cut-off of 0.67 using the '--grm-cutoff' flag.
392 We ran the analyses for three traits per species: intertegular distance (ID), glossa length
393 (GL), and the residuals from a linear regression of GL~ID to control for body size. The GRM
394 was included as a polygenic/random effect. The analysis assumes phenotypes are normally
395 distributed and so samples were removed in the tails of the distributions to meet this
396 assumption for all traits except intertegular distance in *B. sylvicola*, which was normally
397 distributed. For *B. balteatus*, this resulted in 279, 291, and 262 samples for the glossa
398 length, intertegular distance, and residuals analyses respectively. For *B. sylvicola* there were
399 176, 192, and 152 samples for the glossa length, intertegular distance, and residuals
400 analyses respectively. We assessed divergence of p-values from neutral expectations using
401 QQ-plots and carried out Bonferroni correction of p-values using the 'p.adjust' function in R
402 v4.0.2 to assess genome-wide significance.
403

404 **Genome-wide complex trait analysis**

405 We used GCTA (Yang et al., 2011) to perform genome-wide complex trait analysis. We
406 estimated the phenotypic variance explained by the genome-wide SNPs using genome-
407 based restricted maximum likelihood (GREML). The analysis was performed separately for
408 each species on measures of intertegular distance, glossa length, and glossa length with

409 intertegular distance included as a covariate to control for body size. We ran each analysis
410 twice, once using a GRM based on all samples and a second time with the same GRM
411 produced above where one from each pair of sisters was removed. We calculated 95%
412 confidence intervals around each estimate by multiplying the standard error by 1.96. We
413 used R functions provided in (Wang & Xu, 2019) as well as the GCTA-GREML power
414 calculator (Visscher et al., 2014) to perform power analysis and estimate the effect sizes we
415 can expect to detect with our population datasets.
416
417 **RESULTS**
418 **Generation of a highly-contiguous genome assembly of *B. balteatus***
419 We generated a *de novo* genome assembly for the species *B. balteatus* using Oxford
420 Nanopore (ONT) long-read and 10x Chromium linked-read sequencing and compared this to
421 the assembly for *B. sylvicola* that we recently published (Christmas et al., 2021). Assembly
422 statistics for BBAL_1.0 (*B. balteatus*) and BSYL_1.0 (*B. sylvicola*) are presented in table 1,
423 which includes statistics for the assemblies of *B. terrestris* (BTER_1.0) and *B. impatiens*
424 (BIMP_2.2) for comparison. Total assembly sizes of 250.1 and 252.1 Mbp for *B. balteatus*
425 and *B. sylvicola* respectively are both above the average assembly size of *Bombus* genomes
426 assembled to date of 247.9 Mbp (22 genomes, range 229.8 – 282.1 Mbp) (Heraghty et al.,
427 2020; Sadd et al., 2015; Sun et al., 2020). Contig N50s of 8.60 and 3.02 for BBAL_1.0 and
428 BSYL_1.0 respectively are the longest and third longest contig N50s of all published *Bombus*
429 assemblies, demonstrating these to be highly contiguous assemblies. Megabase-scale contig
430 N50 are typical of long-read assemblies, such as the ones presented here and in Heraghty et
431 al. (2020), and are significantly more contiguous than those based on short-read sequencing
432 technologies such as BTER_1.0 and BIMP_2.2.
433
434 High genome completeness and accuracy is also reflected in the BUSCO analysis, with scores
435 of 99.0% (98.7% single copy, 0.3% duplicated, 0.5% fragmented, 0.5% missing) and 98.2%
436 (97.9% single copy, 0.3% duplicated, 0.4% fragmented, 1.4% missing) reported for BBAL_1.0
437 and BSYL_1.0 respectively. Whole genome alignments to the *B. terrestris* assembly (Sadd et
438 al., 2015) resulted in 93.9% and 91.1% of the *B. balteatus* and *B. sylvicola* genomes being
439 placed on 18 pseudochromosomes. Our annotation pipeline annotated 11,711 and 11,585
440 genes in BBAL_1.0 and BSYL_1.0 respectively. These are highly comparable to the 11,874

441 genes in the *B. terrestris* gene set (v. 1.0), but lower than the 12,728 genes in the *B.*
442 *impatiens* gene set (v. 2.1). Gene annotation of the *Bombus* assemblies presented in
443 (Heraghty et al., 2020; Sun et al., 2020) also resulted in a greater number of genes
444 annotated, ranging from 13,325 to 16,970 genes. BUSCO analyses of the annotated genes in
445 our assemblies do suggest that we are missing some genes from our annotations (complete
446 BUSCO genes in BBAL_01 and BSYL_01 respectively: 86.6% and 86.9%). Repeat content of
447 our assemblies is comparable to that of other *Bombus* assemblies, with 17.11% of BBAL_1.0
448 and 17.85% of BSYL_1.0 identified as repetitive by RepeatMasker (full details of repeat
449 classes in supplementary table S3). These proportions are comparable to the repeat content
450 inferred in the *B. terrestris* and *B. impatiens* genomes.
451
452 **Population resequencing indicates three species clusters, with little population structure**
453 **within species**
454 The locations of sample collections, which comprise 580 samples from across seven
455 localities in the Rocky Mountains, CO, USA, are presented in Fig. 1 (full details in table S1,
456 S2). Illumina sequencing resulted in an average genome coverage of 13.1x per sample, with
457 4.5 and 3.5 million SNPs identified in *B. balteatus* and *B. sylvicola* samples respectively
458 (supplementary table S4). Neighbor-joining trees produced from samples of both groups of
459 samples indicate that the *B. balteatus* samples form a single cluster whereas the *B. sylvicola*
460 samples form two clusters (Fig 1). We previously inferred the largest cluster to be comprised
461 of *B. sylvicola* samples whereas the second cluster is comprised of the cryptic species
462 "incognitus" (Christmas et al., 2021). Among samples originally designated as *B. sylvicola,*
463 24% (n=67) are identified as "incognitus" whereas the remaining 76% (n=214) are *B.*
464 *sylvicola. Bombus balteatus* (n=299) was the most commonly observed species across all
465 localities. The proportions vary between localities (table S1). "Incognitus" samples were
466 collected on six out of the seven mountains, whereas *B. balteatus* and *B. sylvicola* were
467 found on all seven.
468
469 We calculated a pairwise kinship coefficient between samples to infer degrees of
470 relatedness (Danecek et al., 2011; Manichaikul et al., 2010). We inferred a set of samples
471 that were likely to be nestmates (sisters) based on them having the same maternal and
472 paternal parents, under the realistic assumption that each colony is headed by a single

473 monandrous female queen (Estoup et al., 1995). Those identified as sisters were always
474 collected foraging on the same mountain and at the same or immediately neighboring site
475 (table S1). This suggests that foraging bees were generally found in the immediate vicinity of
476 their nests. Among the 299 *B. balteatus* samples, we identified 43 samples from 17 colonies
477 that had another nestmate in the dataset, indicating that we sampled a total of 273
478 colonies. For the 214 *B. sylvicola*, we identified 14 samples from 7 colonies that met this
479 criterion, indicating a total of 207 colonies. For 67 "incognitus", we identified two samples
480 from the same colony, indicating that we sampled 66 colonies.
481
482 We investigated the presence of population genetic structure within each species using
483 several methods. Neighbor-joining trees (Fig. 1) do not reveal the presence of any
484 substructure related to geography for *B. balteatus* or "incognitus". For *B. sylvicola*, all of the
485 samples from Quail Mountain (the furthermost southwestern site) cluster together, but do
486 not separate from the rest on the tree. Principal component analysis further reflected the
487 extremely low level of geographical structure across all species (Fig. 2). One slight exception
488 to this trend is the Quail Mountain population of *B. sylvicola* which, shows a slight degree of
489 separation from the other samples at the positive extreme of PC1 (Fig. 2B), although PC1
490 explains only 1.17% of the variance in the data. Outlier clusters seen in the PCA plots of *B.*
491 *balteatus* and "incognitus" (Fig. 2A,C, circled) are composed of highly related individuals as
492 shown in the kinship analysis, suggesting that the effect of kinship overrides any signal of
493 genetic structure. After removing one of each pair of sisters, samples of the same species
494 cluster tightly together and very little variance is explained by PC1 (Fig S1).
495
496 We assessed connectivity between colonies on different mountains by analyzing the
497 association between kinship and geography using Mantel tests after removing sisters from
498 the datasets (Fig. 3). We found no significant relationship between geographic distance and
499 relatedness for *B. balteatus* (Mantel R = -0.03 (95% ci: -0.04 - -0.02), p value = 0.11; Fig.
500 3A,D). There were small but significant negative relationships for *B. sylvicola* (Mantel R = -
501 0.06 (95% c.i. -0.07 - -0.04), p value = 0.0001; Fig. 3B,E) and "incognitus" (Mantel R = -0.04
502 (95% ci: -0.06 - -0.03), p value = 0.029; Fig. 3C,F), demonstrating greater relatedness at
503 closer locations. In these species, Mantel correlograms indicate significant associations
504 between kinship and geographical distance only at shorter distances (from the same or

505 neighboring mountains), which are no longer significant at greater distances (Fig. 3E-F).
506 These results indicate that the limited population structure is mainly driven by the
507 occurrence of a few highly-related samples from the same localities in the dataset.
508
509 **Decrease in tongue length is ongoing in all species**
510 We made morphological measurements for each sample (table S2). Intertegular distance is
511 considered a proxy for body size (Cane, 1987). We also measured the length of two parts of
512 the tongue: glossa and prementum. These two tongue measures are correlated in all three
513 species (*B. balteatus,* Pearson's r = 0.45, p < 0.001; *B. sylvicola,* Pearson's r = 0.38, p < 0.001;
514 incognitus, Pearson's r = 0.53, p < 0.001, Figs. S2-4). Only glossa is considered further, as it
515 was measured by Miller-Struttmann et al. (2015) and is the tongue part with most relevance
516 to feeding (Cariveau et al., 2016). Glossa is also significantly correlated with intertegular
517 distance (*B. balteatus,* Pearson's r = 0.41, p < 0.001; *B. sylvicola,* Pearson's r = 0.26, p <
518 0.001; incognitus, Pearson's r = 0.56, p < 0.001, Figs. S2-4).
519
520 A comparison of phenotypes between species shows that glossa length is substantially
521 longer in *B. balteatus*, consistent with previous knowledge (ls-means = 4.72 mm, 3.34 mm,
522 and 3.40 mm for *B. balteatus*, *B. sylvicola* and "incognitus" respectively; Tukey test, t ratio =
523 12.25 and 21.17, p < 0.001 for *B. balteatus* compared to *B. sylvicola* and "incognitus"*;* Fig.
524 4A). Average glossa length is extremely similar in *B. sylvicola* and "incognitus" (Tukey test, t
525 ratio =0.50, p = 0.87), suggesting that this character does not distinguish this newly-
526 discovered species from *B. sylvicola*. *Bombus balteatus* bees are significantly larger bees as
527 indicated by a greater intertegular distance, and *B. sylvicola* is significantly larger than
528 "incognitus" (mean intertegular distance = 4.00 mm, 3.83 mm, 3.58 mm for *B. balteatus*, *B.*
529 *sylvicola* and "incognitus" respectively; ANOVA, F=28.75, p < 0.001, Tukey post-hoc test
530 revealed differences are significant among all species at p < 0.001; Fig. S5A).
531
532 We next compared the distribution of glossa lengths in our samples with historical data. We
533 combined *B. sylvicola* and "incognitus" for this analysis because they do not differ in glossa
534 length on average and they were not distinguished in previous studies. We find an overall
535 trend of decreasing glossa length in both *B. sylvicola*-incognitus and *B. balteatus* over time
536 (Fig. 4B,C). This was reported previously between the collections from 1966-1980 and from

537 2012-2014 for both species (Miller-Struttmann et al., 2015). Here we show that this trend is
538 continuing, with further reductions in glossa length between the 2012-2014 collections and
539 our collections from 2017. These reductions are significant for both *B. sylvicola*-incognitus
540 (ls-means: 2012-2014 = 3.51 mm, 2017 = 3.21 mm; Tukey test, t-ratio = 3.198, p = 0.004)
541 and *B. balteatus* (ls-means: 2012-2014 = 5.24 mm, 2017 = 4.84 mm; Tukey test, t-ratio =
542 2.79, p = 0.015). In contrast, there is no significant trend shown by intertegular distance (Fig.
543 S5B,C), although for *B. sylvicola*-incognitus*,* intertegular distance is significantly larger in
544 both the 1966-80 and 2017 samples compared to the 2012-2014 samples (ANOVA, F =
545 15.28, p < 0.001. Tukey post-hoc test, p < 0.001).
546
547 For samples collected in 2017, we compared the distribution of glossa lengths for each
548 species among the seven mountains where they were collected (Fig. S6). There is an overall
549 trend for glossa lengths to be longer in bees from Mount Evans than on other mountains in
550 all species. The distributions for *B. balteatus* and *B. sylvicola* show significant differences,
551 with samples from Mount Evans having significantly longer glossae than on Mount
552 Democrat, Horseshoe Mountain, and Pennsylvania Mountain in *B. balteatus* (ls-mean glossa
553 length on Mt. Evans = 5.34 mm, other mountains 4.51 – 4.73 mm; Tukey p < 0.01 in each
554 case; Fig. S6A) and significantly longer glossae than on all other mountains except Niwot
555 Ridge in *B. sylvicola* (ls-mean glossa length on Mt. Evans = 3.77 mm, other mountains 3.02 –
556 3.24 mm; Tukey p < 0.01 in each case; Fig. S6B). A similar but non-significant trend could
557 also be seen for "incognitus" (Fig. S6C). Comparisons of intertegular distance between
558 mountains revealed that *B. balteatus* bees sampled on Niwot Ridge are significantly larger
559 than those on Boreas Mountain, Mount Democrat, Horseshoe Mountain, and Quail
560 Mountain (Niwot Ridge mean intertegular distance = 4.25 mm, other mountains 3.84 - 4.00
561 mm, ANOVA, F = 4.09, p < 0.001, Tukey post-hoc test p < 0.05 in four comparisons; Fig. S7A).
562 We did not identify any significant differences in intertegular distance of bees across
563 mountains for *B. sylvicola* (ANOVA, F = 1.83, p = 0.10) or "incognitus" (ANOVA, F = 1.62, p =
564 0.17; Fig. S7B,C).
565
566 **Genome-wide association study identifies a SNP associated with a proxy for body size**
567 We used a linear mixed-model analysis to search for correlations between allele frequencies
568 and quantitative trait variation in *B. sylvicola* and *B. balteatus.* We used the traits glossa

569 length, intertegular distance and glossa length corrected for intertegular distance (see
570 Methods). One from each pair of sisters identified in the kinship analysis was removed from
571 all analyses to reduce the level of relatedness in the sample set. We detected a genome-
572 wide significant association between intertegular distance and a SNP in intron 9 of the
573 *CTRB1* (Chymotrypsinogen B1) gene on chromosome 10 in *B. sylvicola* ($p < 0.001$ after
574 Bonferroni correction; Fig. 5). The SNP is found at an allele frequency of 0.14 across all
575 populations, with 41 individuals heterozygous and nine homozygous for the allele. The allele
576 is present on all mountains, ranging in allele frequency from 0.06 (Quail Mountain) to 0.24
577 (Niwot Ridge). The *CTRB1* gene is involved in production of chymotrypsin, a proteolytic
578 digestive enzyme (Burgess, Malone, & Christeller, 1996; Giebel, Zwilling, & Pfleiderer, 1971;
579 Grogan & Hunt, 1979; Lazarević & Janković-Tomanić, 2015; Matsuoka et al., 2015; Rawlings
580 & Barrett, 1994). Intron 9 is a large 18 Kbp intron and the significant SNP is located 2 Kbp
581 upstream of exon 10. The GWAS significance suggests it is of likely functional importance
582 and its location is suggestive of a regulatory element, such as an intron splice enhancer or
583 silencer.
584
585 Two further SNPs, located 4 bp apart on chromosome 11, also significantly associated with
586 intertegular distance ($p = 0.039$ after Bonferroni correction). However, they are not located
587 in or close to any genes according to our annotation, so any potential functional significance
588 of these SNPs is unknown. We did not find any significant associations between allele
589 frequencies and intertegular distance in *B. balteatus* (Fig. S8). Furthermore, no significant
590 associations were found between allele frequencies and glossa length, or glossa length
591 corrected for intertegular distance in either species (Fig. S8). This indicates that the trait has
592 a polygenic or mainly environmental component. A power analysis (Visscher et al., 2014)
593 revealed that our datasets have sufficient power (>0.8) to detect a QTL with a h^2 of ~0.1 but
594 that loci with lower effect sizes would likely be undetectable.
595
596 **Limited evidence that morphological variation has a genetic component**
597 We performed GCTA-GREML analyses (Yang et al., 2011) to determine the genetic
598 component of trait variation for *B. sylvicola* and *B. balteatus* (Fig. 6, supplementary table
599 S5). The "incognitus" samples were not included here due to smaller sample size. When
600 each species' entire dataset is considered, we detect a heritable component for both glossa

length and intertegular distance in both species, where the 95% confidence interval does not overlap zero. For *B. sylvicola*, but not *B. balteatus*, this is also the case when intertegular distance is introduced as a covariate to account for body size when considering glossa length. However, when we remove samples that we infer to be workers from the same colony from the kinship analysis, we only detect a heritable component with 95% confidence for intertegular distance in *B. balteatus* (all others then overlap with zero). This indicates that a large proportion of the signal of covariance between genetic and trait variance is due to the presence of sisters from the same colony. This could be a confounding factor because environmental effects such as level of nutrition and colony health are likely to covary with colony. However, such covariation could also reflect genetics. The level of dependency of phenotype on genotype is therefore unclear from this analysis.

The 95% confidence intervals for the estimate of heritability have a wide span and overlap zero in many cases. The power to determine heritability accurately depends on several factors, including sample size, the structure of relatedness in the samples, and the true value of heritability. When samples are all completely unrelated, it has been estimated that > 3,000 samples are required to achieve a SE below 0.1 (Visscher et al., 2014). For datasets of unrelated samples of the size we have analyzed here, we estimate a power of ~0.06, or ~6% probability, to detect $h^2 > 0$ for a trait with a SNP-heritability of 0.5 and a type 1 error rate (α) of 0.05. Greater sample size or higher levels of relatedness within the dataset would therefore increase accuracy of our heritability estimates.

DISCUSSION

We generated a high-quality reference genome and performed genome sequencing of a population sample of the bumblebee species *B. balteatus*. Combining these data with a previous dataset from *B. sylvicola* produced the most extensive population genomics dataset for bumblebees to date, with 580 samples from these two species and the newly-discovered “incognitus” collected from high-altitude locations in Colorado, USA. These data were combined with morphometric measurements from each sample and used to investigate genetic structure, morphological evolution and the genetic basis of phenotypic traits in these populations. Our main findings are 1) no evidence for gene flow between species, including the recently-inferred "incognitus", but lack of population structure within

species, indicating high levels of connectivity among subpopulations. 2) An ongoing decrease in tongue (glossa) length compared to historical measurements, previously inferred to reflect the effects of decreasing floral abundance on bumble bee foraging (Miller-Struttmann et al., 2015). 3) An association between body size and variation at a single SNP located in a gene related to production of a digestive enzyme in *B. sylvicola*. 4) A lack of conclusive evidence for a genetic component to tongue length in both species. Our analysis is unable to determine the degree of heritability underlying tongue length or body size but is consistent with these traits having a polygenic genetic component.

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

Identification of the previously-unknown species “incognitus” demonstrates the power of population-scale genome sequencing to discover new cryptic variation (Christmas et al., 2021). None of the morphological features that we studied could distinguish between *B. sylvicola* and “incognitus”. *Bombus sylvicola* did however have a significant tendency to be collected at higher altitudes and have larger body size. The wider distribution of “incognitus” is unknown, as is the true composition of populations described as *B. sylvicola* in other geographical locations. Existing collections of *B. sylvicola* from Colorado are likely to be a mixture of both species. However, importantly for this study, there were no significant differences in glossa or prementum length between the two species.

We find no evidence for population structure among samples of any of the three species studied here. This indicates an absence of long-term geographical barriers to gene flow among the high-elevation localities included here. Furthermore, although we observe

elevated degrees of relatedness among foragers collected on the same mountain, there is no significant tendency for elevated relatedness of foragers caught on nearby mountains, indicating a lack of population structure at this scale. Foragers inferred to belong to the same nest (i.e. with identical parents) were always found in the same or adjacent sites, consistent with the limited foraging distance (on average < 110m) inferred previously by Geib et al. (2015) for these species.

Studies of several bumblebee species across continental USA, including *B. bifarius* and *B. vosnesenkii*, also found in Colorado and across western North America, indicate weak geographical differentiation even at distances over 1000 km (Ghisbain et al., 2020; Lozier et al., 2011). However, the complex topography of mountain ranges likely modulates gene flow among populations (Lozier et al., 2013, 2011). Populations of *B. bifarius* are inferred to be more fragmented in the southern portions of their ranges, where they occur at higher elevations (Jackson et al., 2018). The species studied here, *B. sylvicola* and *B. balteatus,* are generally restricted to high-elevation localities above the tree line separated by forest (Williams, Thorp, Richardson, & Colla, 2014). However, our analysis indicates that this complex landscape has not historically limited mating and dispersal between localities in the same mountain range. This is consistent with previous studies of these species that found substantial genetic differentiation between mountain ranges in the Pacific Northwest, but not within the same mountain range (Koch et al., 2017; Whitley, 2018).

Morphological change over time has already been documented in the species studied here, with a significant reduction in tongue length observed since 1966 (Miller-Struttmann et al., 2015). During this time period, there has also been a shift in species composition, with shorter-tongued species becoming more predominant. Previous collections of *B. sylvicola* in this region are likely a mixture of *B. sylvicola* and “incognitus”, as the existence of “incognitus” was unknown. However, as *B. sylvicola* and “incognitus” do not differ in glossa length, undetected changes in the relative abundance of these species over time are unlikely to be responsible for the observed shifts in glossa length observed in samples that are defined as *B. sylvicola.* This effect could, however, potentially contribute to the observed variation in body size over time as *B. sylvicola* have a significantly greater intertegular distance on average than “incognitus” (Fig. S5).

A major finding that we present here is that the temporal shift toward shorter tongue length is ongoing. In addition to the significant reduction previously observed over a period of six decades up until 2014, we also observe a significant reduction over a much shorter time period between 2012-14 and 2017 in both *B. balteatus* and *B. sylvicola*-incognitus. Mean tongue length reportedly decreased on average by 0.61% annually in a period between 1966 and 2014 for *B. sylvicola* (Miller-Struttmann et al., 2015). According to our recent measurements, the corresponding decrease in 3-5 subsequent years has been 12.9 %, or 3.2% per year (compared to the 2012-14 average).

Decreases in tongue-length have been previously attributed to an advantage of shorter tongues for foraging on a wide range of flowers. Such generalist foraging could be favored due to a general decline of floral resources observed in connection with warmer summers (Miller-Struttmann et al., 2015). While both long- and short-tongued bees are capable of accessing nectar in plants with short flower tubes, short-tongued bees are more efficient at foraging from them (Inouye, 1980) and are also more efficient at foraging across a diversity of flower tube depths (Arbulo, Santos, Salvarrey, & Invernizzi, 2011; Geib & Galen, 2012; Plowright & Plowright, 1997). Short-tongued bumble bees often forage from a wider suite of plants both in these alpine bumble bee species (Miller-Struttmann et al., 2015) and others (Goulson & Darvill, 2004; Goulson et al., 2008b; Heinrich, 2004; Huang et al., 2015). These considerations indicate that shorter tongues are likely advantageous for generalist foraging. Although short-tongued bees are unable to pollinate plants with long flower tubes, they are likely important for supporting a diverse array of flowering plants.

It is unclear whether the tongue-length changes we observe are the result of natural selection or phenotypic plasticity. There are now many examples of rapid evolution of traits driven by natural selection due to climatic events in the wild, such as a shift in beak shape over a period of a few years in Darwin's finches related to competition for food during drought (Lamichhaney et al., 2016) and genomic shifts related to cold-tolerance in the green anole lizard related to severe winter storms (Campbell-Staton et al., 2017). Human activity may also promote rapid adaptation by natural selection, as demonstrated by the evolution of longer beaks in great tits promoted by the use of bird feeders in the UK (Bosse et al.,

729 2017). Global climate change is also expected to be a driver of rapid evolution, but it is often
730 difficult to establish whether trends of phenotypic change result from evolution or
731 phenotypic plasticity (Merilä & Hendry, 2014). Long-term changes in breeding timing in
732 great tits (Charmantier et al., 2008) and body mass in red-billed gulls (Teplitsky, Mills, Alho,
733 Yarrall, & Merilä, 2008) that track temperature increases have been shown to result solely
734 from phenotypic plasticity.
735
736 To address the causes of the changes in bumblebee tongue length observed here, we
737 attempted to uncover the genetic basis of morphological variation using genome-wide
738 association studies (GWAS) and genome-wide complex trait analysis (GCTA). No significant
739 associations were observed for tongue length in the genome of any of the species using
740 GWAS. However, a significant association with body size was identified in *B. sylvicola* at a
741 single SNP in the *CTRB1* (Chymotrypsinogen B1) gene. The lack of association at neighboring
742 SNPs could be attributable to high rates of meiotic recombination and low levels of linkage
743 disequilibrium in bumblebees (Kawakami et al., 2019). The *CTRB1* gene produces a
744 precursor of chymotrypsin, a key digestive enzyme in animals, which is likely important for
745 pollen digestion in bees (Burgess et al., 1996; Giebel et al., 1971; Grogan & Hunt, 1979;
746 Lazarević & Janković-Tomanić, 2015; Matsuoka et al., 2015; Rawlings & Barrett, 1994). The
747 associated SNP is found in a large intron (18 Kbp) of this gene, 2 Kbp upstream of the
748 nearest exon. We lack any functional annotation for this SNP but its location and GWAS
749 significance suggests it may play a role in gene regulation. For example, if located within an
750 intronic splicing enhancer or silencer, it may result in variation in expression levels of
751 different isoforms of the CTRB1 enzyme being produced. Such changes could influence body
752 size through an effect on the efficiency of digestion.
753
754 We detected a significant signal of covariance of trait measurements with relatedness for
755 both tongue length and intertegular distance using GCTA when closely-related individuals
756 were included in the dataset. However, no significant signal of heritability in morphological
757 traits was detected when the dataset was purged of close relatives. The first result could
758 potentially reflect a genetic component, but it is not possible to disentangle this component
759 from the shared environmental effects due to close relatives sharing a nest. This analysis is
760 therefore underpowered to detect genetic effects due to low sample size and a general lack

761 of related individuals in the dataset. A possible explanation for our results is that the
762 morphological traits tongue length and intertegular distance have a highly polygenic genetic
763 component, with multiple QTL of small effect ($h^2 < 0.1$), such that GWAS and GCTA were
764 underpowered to detect QTL and estimate heritability. The size of the genetic component of
765 variation in both traits is therefore still unknown in each species. These results contrast with
766 findings regarding the genetic control of bumblebee coloration, in which variation at single
767 loci control intraspecific variation in color of abdominal segments (Rahman, Cnaani, Kinch,
768 Grishin, & Hines, 2021; Tian et al., 2019).
769
770 It therefore remains unclear whether the observed changes in tongue length are caused by
771 natural selection acting on functional genetic variation, or whether they are the result of
772 phenotypic plasticity. There is large variation in body size between individuals in bumblebee
773 nests, indicating substantial phenotypic plasticity in this trait (Peat, Tucker, & Goulson,
774 2005). This variability could be explained by a correlation between larval position in the nest
775 and size, which is related to feeding intensity (Couvillon & Dornhaus, 2009). There is
776 evidence from solitary bees of an effect of temperature on body size (Scaven & Rafferty,
777 2013), although such an effect is likely to be less pronounced in social insects such as
778 bumblebees that regulate the temperature of their nests. However, it is unclear whether
779 tongue length also exhibits plasticity, as this trait correlates with body size among
780 nestmates (Peat et al., 2005), indicating that plasticity in tongue length independent of body
781 size is less pronounced. However, our observation of significant differences in mean tongue
782 length between mountains could potentially reflect phenotypic plasticity.
783
784 In summary, we have shown that decreasing tongue length observed over the last six
785 decades is still ongoing in populations of the bumblebee species *B. sylvicola* and *B. balteatus*
786 (and the recently-reported "incognitus") at high altitudes in Colorado. We have generated
787 the genomic resources to study these species on the genetic level in the form of highly-
788 contiguous genome assemblies and comprehensively characterized genome variation. Our
789 results indicate that the complex topology of the mountain landscape in Colorado does not
790 contain significant barriers to mating and dispersal of bumblebees in the study area. We are
791 unable to conclude whether the observed morphological shifts over time are the result of
792 genetic adaptation or phenotypic plasticity. Alpine ecosystems are particularly sensitive to

climate change and may suffer its effects before other geographical regions (Elsen & Tingley, 2015). Continued monitoring of changes in morphology in these and other populations in these regions could be highly valuable for understanding how pollinators respond to the effects of a warming climate, the degree to which rapid evolution by natural selection or phenotypic plasticity are possible, and the effects of the changes on these ecosystems.

DATA AND CODE AVAILABILITY

All sequence data presented here are available at NCBI under BioProject IDs PRJNA646847 and PRJNA704506. Scripts and annotation parameters used in the study are available at GitHub (https://github.com/matt-webster-lab/alpine_bumblebees). VCF files containing genotypic variation are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.mgqnk990p).

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AUTHOR CONTRIBUTIONS

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Figure 1. Population sampling and genetic variation in three *Bombus* species. (A) Map showing the sampling locations of *Bombus balteatus, B. sylvicola,* and "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 "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) "incognitus" populations. Numbers in brackets on axes indicate the percentage variance explained by each principal component.

Figure 3. Correlations between kinship and geographic distance. Geographic distance between all pairs of samples plotted against kinship coefficient (a measure of relatedness) between pairs are shown for (A) *Bombus balteatus*, (B) *B. sylvicola*, and (C) "incognitus", where each point represents a pair. (D-F) Mantel correlograms showing the Mantel correlation between kinship and geographic distance over 10 km distance classes for (D) *Bombus balteatus,* (E) *B. sylvicola*, and (F) "incognitus". Filled in boxes indicate significant correlations ($p < 0.01$). There were insufficient data points for calculating Mantel correlations at distance classes > 60 km for *B. balteatus* and *B. sylvicola*. For "incognitus", distance class 10-20 km as well as all distance classes > 50 km lacked sufficient data.

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, correcting for body size and sample location, per sampling period for (B) *B. balteatus* and (C) *B. sylvicola* and "incognitus" combined. 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. (A) 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 balteatus* pseudochromosomes. Red dashed line indicates genome-wide significance after Bonferroni correction for $p = 0.01$. A single SNP on chromosome 10, coloured and circled in 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. (B) Zoomed-in plot of the significant SNP in chromosome 10 showing its location within an intron of the *CTRB1* gene. Exons of this gene are represented by purple boxes, introns are represented by dashed purple lines.

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 intertegular distance 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), to assess the effect of including highly related samples in the analysis.

Table 1: Assembly metrics for *B. balteatus*, with three 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

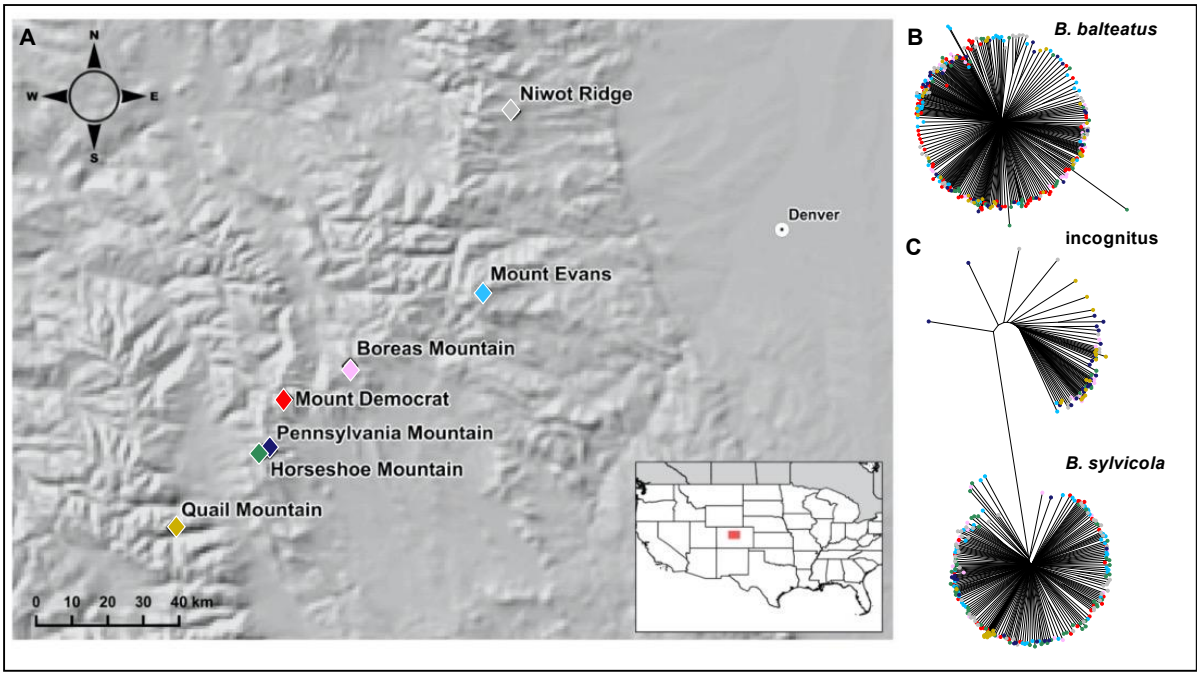
A
N
W
E
S
Niwot Ridge
Denver
Mount Evans
Boreas Mountain
Mount Democrat
Pennsylvania Mountain
Horseshoe Mountain
Quail Mountain
0 10 20 30 40 km
B
B. balteatus
C
incognitus
B. sylvicola

Figure 1

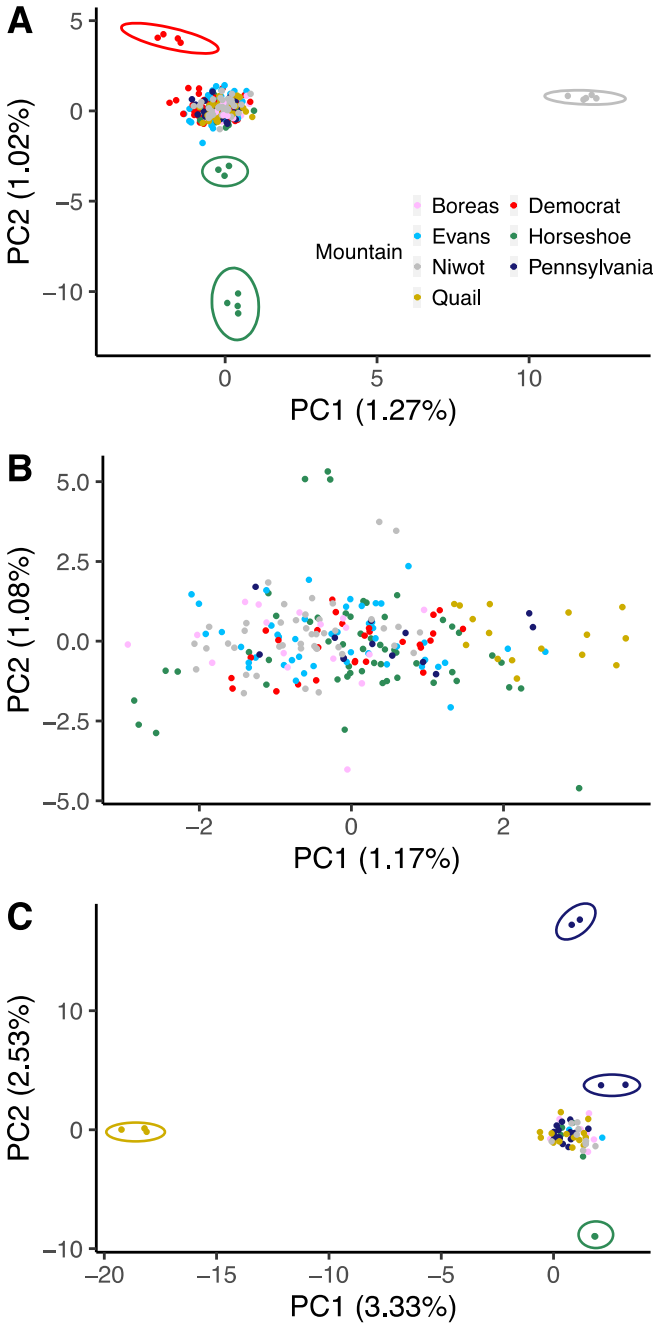
A
PC2 (1.02%)
PC1 (1.27%)
Mountain
Boreas
Evans
Niwot
Quail
Democrat
Horseshoe
Pennsylvania
B
PC2 (1.08%)
PC1 (1.17%)
C
PC2 (2.53%)
PC1 (3.33%)

Figure 2

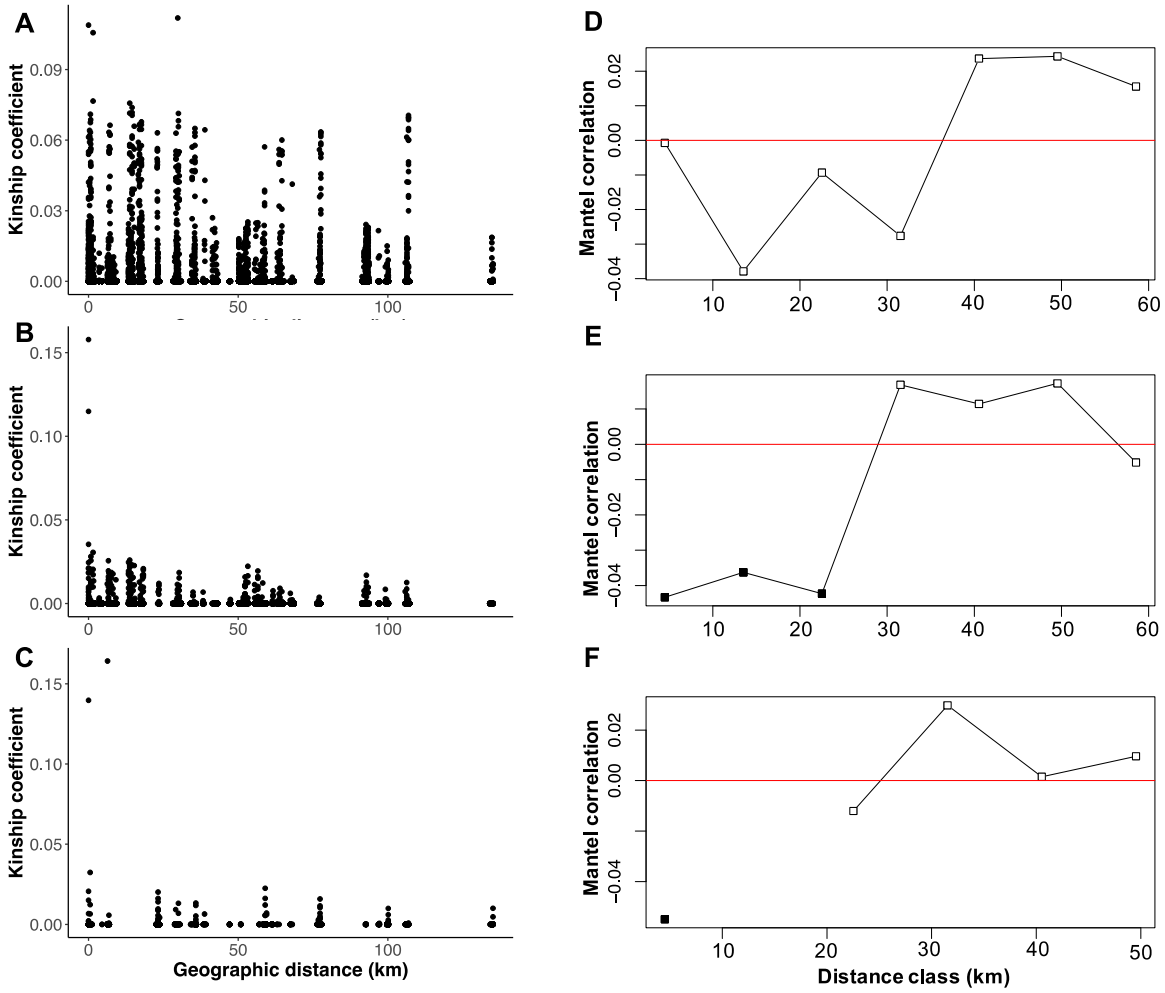
A
B
C
D
E
F
Kinship coefficient
Mantel correlation
Geographic distance (km)
Distance class (km)

Figure 3

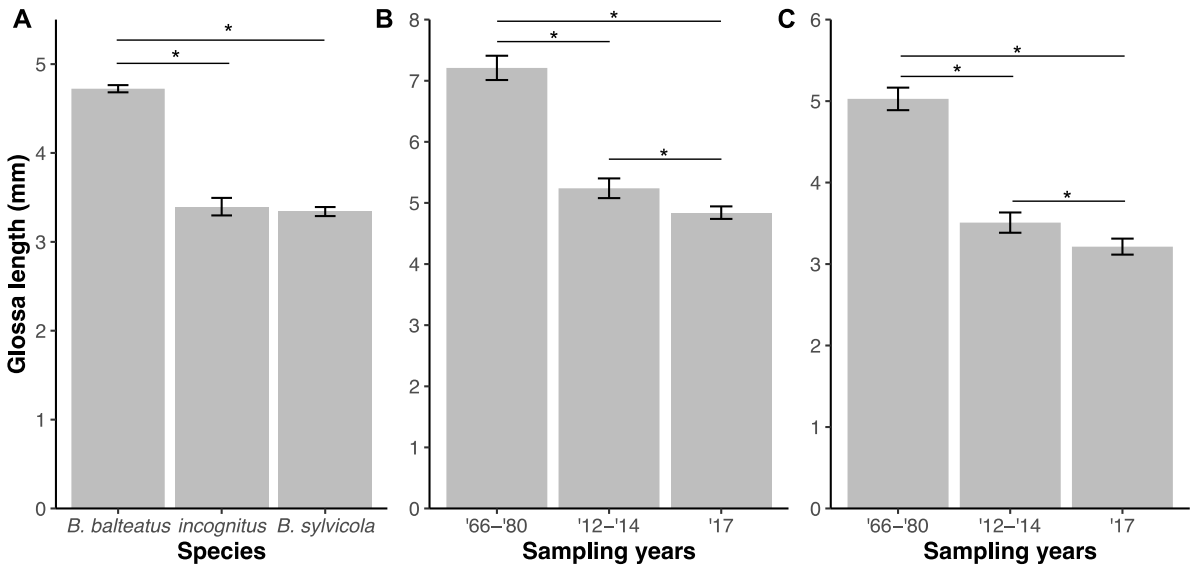
A
B
C
Glossa length (mm)
B. balteatus
incognitus
B. sylvicola
Species
'66–'80
'12–'14
'17
Sampling years
Sampling years

Figure 4

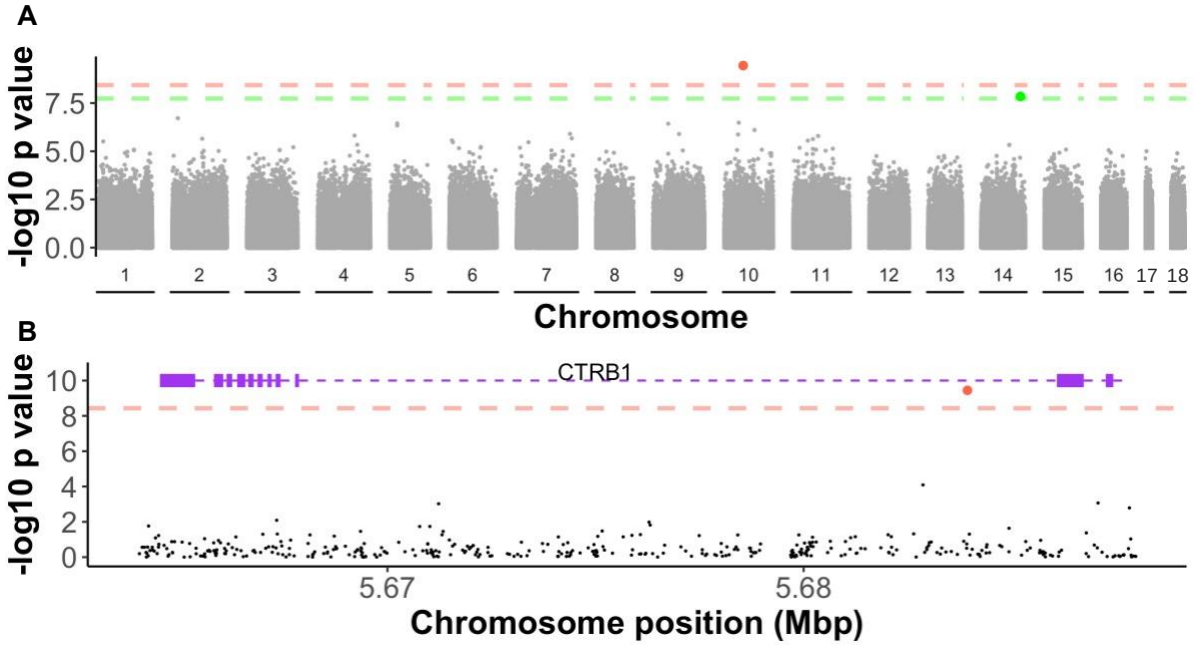
A
-log10 p value
7.5
5.0
2.5
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Chromosome
B
-log10 p value
10
8
6
4
2
0
CTRB1
5.67
5.68
Chromosome position (Mbp)

Figure 5

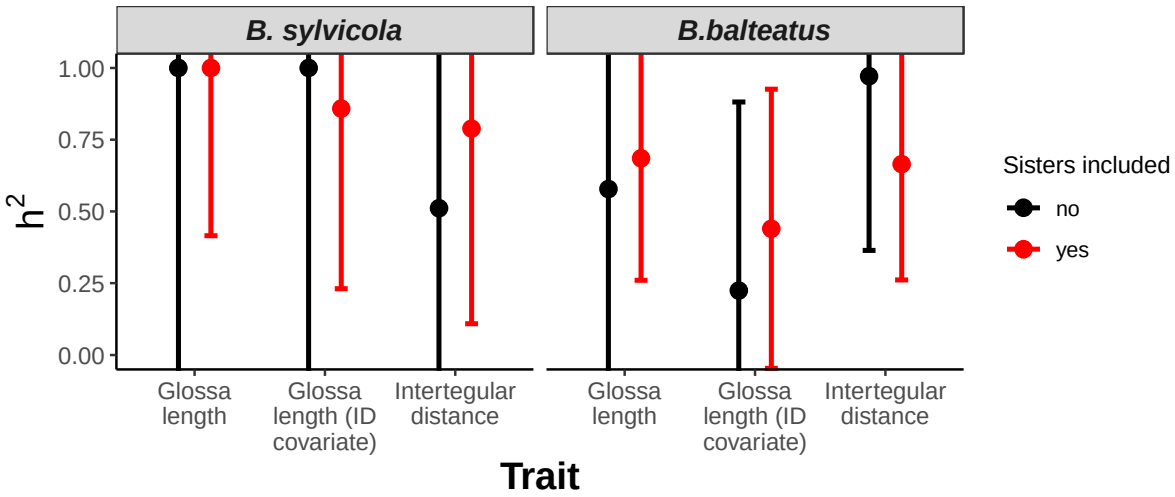
B. sylvicola
B.balteatus
1.00
0.75
0.50
0.25
0.00
h2
Glossa length
Glossa length (ID covariate)
Intertegular distance
Glossa length
Glossa length (ID covariate)
Intertegular distance
Trait
Sisters included
no
yes

Figure 6