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

Matthew J. Christmas[1], Julia C. Jones[1,2], Anna Olsson[1], Ola Wallerman[1], Ignas Bunikis[3], Marcin Kierczak[4], Kaitlyn M. Whitley[5,6], Isabel Sullivan[5,7], Jennifer C. Geib[5], Nicole E. Miller-Struttmann[8], Matthew T. Webster[1]

1) Department of Medical Biochemistry and Microbiology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden
2) School of Biology and Environmental Science, University College Dublin, Dublin, Ireland
3) Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden
4) Department of Cell and Molecular Biology, National Bioinformatics Infrastructure Sweden, Science for Life Laboratory, Uppsala University, Uppsala, Sweden
5) Department of Biology, Appalachian State University, Boone, North Carolina, USA
6) U.S. Department of Agriculture, Agriculture Research Service, Charleston, South Carolina, USA
7) Marine Estuarine Environmental Sciences, University of Maryland, College Park Maryland
8) Biological Sciences Department, Webster University, St. Louis, Missouri, USA

Corresponding author: Matthew T. Webster
Address: Dept. Medical Biochemistry and Microbiology, Box 582, 75123 Uppsala, Sweden
Email: matthew.webster@imbim.uu.se
Tel: +46 18 471 4391

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 use 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 generate a genome assembly of *B. balteatus*. We then analyze 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 find 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 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 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 *et al.* 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 (Goulson *et al.* 2008a; Cameron *et al.* 2011; Cameron & Sadd 2020; Soroye *et al.* 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

59 particularly threatened by these trends, and can be considered "canaries in the coal mine"
60 for detecting early effects (Elsen & Tingley 2015).
61
62 In addition to range shifts there is evidence that the morphology of certain populations of
63 bumblebees has shifted in recent decades. Miller-Struttmann et al. (2015) compared
64 morphometric measurements of contemporary samples and historical specimens of the
65 species *B. balteatus* and *B. sylvicola* from the same high-elevation alpine localities in
66 Colorado. These species historically comprise the majority of bumblebees in these locations
67 (Miller-Struttmann *et al.* 2015). They reported a significant decrease in tongue length in
68 both species in a period between 1966 and 2014. The cause of this was inferred to be a
69 general decrease in floral abundance resulting from warmer temperatures and dryer soils
70 resulting from climate change. These conditions could favor more generalist foraging
71 strategies, which several studies indicate are performed more efficiently by bees with
72 shorter tongues (Heinrich 2004; Goulson & Darvill 2004; Goulson *et al.* 2005, 2008b; Huang
73 *et al.* 2015). Continued monitoring of these populations is important to understand the
74 causes of these morphological shifts and their ecological consequences. It is unknown
75 whether the trend towards shorter tongues has continued in more recent years.
76
77 One explanation for the observed morphological shifts in *B. balteatus* and *B. sylvicola* in
78 Colorado is that natural selection for shorter tongues has occurred (Miller-Struttmann et al.,
79 2015). However, it also possible that the observed changes are a plastic response to
80 environmental change (Merilä & Hendry 2014). It is unknown whether this trait has a
81 genetic component that could be acted on by natural selection. Uncovering the size of the
82 genetic contribution to this trait and the number and identity of any genetic loci involved
83 would be an important step towards understanding the underlying cause of the
84 morphological changes. The feasibility of genome sequencing on a population scale has
85 enabled studies of quantitative genetics (Gienapp *et al.* 2017) and genome-wide association
86 studies (Santure & Garant 2018) in wild populations, allowing these goals to be pursued.
87
88 The genetic structure of populations is an important factor for interpretation of genotype-
89 phenotype correlations and the cause of morphological shifts. We previously presented a
90 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 *et al.* 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 *et al.* 2006; Ellis *et al.* 2006; Jha 2015; Woodard *et al.* 2015; Koch *et al.* 2017; Jackson *et al.* 2018; Ghisbain *et al.* 2020). 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 *et al.* 2011, 2013). 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 (Darvill *et al.* 2004; Knight *et al.* 2005; Jha & Kremen 2013; Geib *et al.* 2015; Woodard *et al.* 2015). These variables show large variation between species and are strongly dependent on

landscape composition. Genome sequencing of multiple individuals has the potential to add greater resolution to our understanding of population density, structure and dispersal.

In this study we present a high-quality annotated genome assembly of the bumblebee species *B. balteatus* based on long-read Oxford Nanopore sequencing. We also conducted whole-genome sequencing of 299 samples of this species sampled from seven high-altitude localities spread across 150 km in Colorado. These localities have an average separation of 54 km between each other, and consist of alpine tundra separated by forest. We analyze these data together with the previously-published population genomic dataset of 284 samples *B. sylvicola* and "incognitus" from the same localities (Christmas *et al.* 2021). We use these data to assess whole-genome variation and its connection to geographical and morphological variation in these populations, which were previously studied by (Miller-Struttmann *et al.* 2015). We then analyzed morphological variation in tongue length and body size in context of previous studies (Miller-Struttmann *et al.* 2015) 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 *et al.* 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.

Our study species have recently been subject to taxonomic revision. The species we refer to here as *B. balteatus* was previously described as occurring in both North America and Eurasia, but a recent study classified these geographically separated populations as two different species: *B. balteatus* in Eurasia and *Bombus kirbiellus* in North America (Williams *et al.* 2019). The species we refer to here as *B. sylvicola* was previously split into a North American form (*B. sylvicola*) and a Eurasian form (*Bombus lapponicus*). However, a recent 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

155 **Sample collection**

156 We collected samples identified as *B. balteatus* and *B. sylvicola* (Koch *et al.* 2014) during the
157 summer of 2017. Bumblebees were collected on seven different mountains in Colorado,
158 USA, with an average distance of 54 km between mountains (greatest distance: 134 km,
159 Quail Mountain – Niwot Ridge; shortest distance: 10km, Mount Democrat – Pennsylvania
160 Mountain). The existence of "incognitus" was unknown to us when the bees were sampled
161 but a large fraction of bees identified as *B. sylvicola* were subsequently assigned to
162 "incognitus" on the basis of genetic analysis (Christmas *et al.* 2021). Three of these
163 mountains (Mount Evans (39.583883, -105.63845), Niwot Ridge (40.05945, -105.616667),
164 and Pennsylvania Mountain (39.263383, -106.142733)) were also sampled previously by
165 (Miller-Struttmann *et al.* 2015). Four additional mountains were sampled here (Boreas
166 Mountain (39.419375, -105.95441), Mount Democrat (39.33721, -106.12581), Horseshoe
167 Mountain (39.204605, -106.181085) and Quail Mountain (39.018158, -106.404877)) but
168 were not sampled in the previous study. Each of the mountain locations comprised several
169 neighboring sampling sites separated by short distances (mean = 1.1 km) and elevations
170 (range: 3,473 – 4,012 m.a.s.l.) (full details in supplementary tables S1, S2).

171

172 We collected samples of foraging worker bees from each site using sweeping hand nets. We
173 kept samples in Falcon tubes in cool boxes for transport. Species assignment was performed
174 by observing standard morphological characters (Koch *et al.* 2014). We dissected each
175 sample after placing it at -20°C for approximately 10 minutes. We stored thoraces in 95%
176 ethanol prior to DNA extraction whereas the heads were stored for morphometric analysis.

177

178 **Morphological measurements**

179 We measured intertegular distance (ID) and tongue length on all of the samples of *B.*
180 *sylvicola,* "incognitus" and *B. balteatus* using scaled photographs of individual bee heads
181 processed by ImageJ (Kearns & Inouye 1993; Schneider *et al.* 2012). Following netting, bees
182 were anesthetized by chilling them on ice or in a freezer (depending on field conditions).
183 Once anesthetized, the bees were removed from the vial, gently pressed prone to the table
184 with the thorax parallel to the camera lens using flat forceps, and photographed with a
185 metric ruler for scaling purposes. The distance between the tegula was measured as a
186 straight line between the edge of one tegula to the other tegula, running as near to

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

218 R9.4 flowcell. This resulted in 23.3 Gpb of sequence data, in 3.9 million reads with a mean
219 length of 5.9 kbp.
220
221 We assembled the ONT reads using the following process: We first used downpore with
222 default parameters for adaptor trimming and splitting chimeric reads. We then assembled
223 the trimmed reads using wtdbg2 (Ruan & Li 2020) on default settings. We then ran two
224 rounds of the consensus module Racon and contig improvements using medaka v.0.4,
225 removing contigs of length < 20 kbp. We used Illumina short-read data from the population
226 sequencing to perform two rounds of Pilon to improve sequence accuracy, particularly
227 around indels.
228
229 We also sequenced the same sample of each species using 10x Genomics Chromium. A 10x
230 GEM library was constructed according to the manufacturer's protocols. We quantitated
231 each library using qPCR and sequenced them together on a single lane of HiSeq 2500 using
232 the HiSeq Rapid SBS sequencing kit version 2, resulting in 150 bp paired-end sequences. The
233 linked reads were mapped to the assembly using Longranger v.2.1.4. We then ran Tigmint
234 v1.1.2 to correct errors in the assembly detected by the read mappings. We removed
235 contigs containing multiple mitochondrial genes identified using BLAST+ v2.9.0. We also
236 removed all contigs shorter than 10 kbp.
237
238 **Genome analysis**
239 BUSCO v3.0.2b (Simão *et al.* 2015) was used to assess the assembly completeness using the
240 hymenoptera_odb9 lineage set and species *B. impatiens*. We ran RepeatMasker v.4.1.0 on
241 each genome assembly to characterize genome repeat content, including interspersed
242 repeats and low complexity sequences. It has been shown that bumblebees have a stable 18
243 chromosome karyotype across all subgenera (Sun *et al.* 2020). We therefore performed
244 whole-genome synteny alignments between the *B. terrestris* chromosome-level genome
245 assembly (Sadd *et al.* 2015) (downloaded from NCBI; BioProject PRJNA45869) and our
246 assemblies using Satsuma v.3 (Grabherr *et al.* 2010) to arrange the contigs from our
247 assemblies onto 18 pseudochromosomes.
248
249 **RNAseq and Annotation**

250 We used the Nextflow pipelines available at https://github.com/NBISweden/pipelines-
251 nextflow for genome annotation. The pipelines, in turn, depend on two additional
252 annotation-specific toolkits, AGAT and GAAS, available
253 at https://github.com/NBISweden/AGAT and https://github.com/NBISweden/GAAS respecti
254 vely. Both repositories contain extensive documentation and installation instructions. As
255 reference proteins database, we used the SwissProt/UniProt (561,356 curated proteins,
256 downloaded 2019-11). The parameter files stated below are available at
257 https://github.com/matt-webster-lab/alpine_bumblebees.
258
259 We performed RNAseq on a single lane of Illumina HiSeq 2500 using samples from four body
260 parts from a single sample of *B. balteatus* (abdomen, head, legs and thorax), which
261 generated 42 Gbp of reads. We performed quality control of the data using FastQC and read
262 trimming using trimmomatic (v. 0.39, trimmomatic.params). We next generated a guided
263 assembly with Hisat2 and Stringtie using the AnnotationPreprocessing.nf (parameters as in
264 AnnotationPreprocessing_params.conf) TranscriptAssembly.nf Nextflow pipeline with
265 parameters as in the TranscriptAssembly_params.config file. In addition to the guided
266 assembly, we performed a de novo assembly for reads from each of the four tissues using
267 Trinity (v. 2.0.4, --seqType fq --SS_lib_type RF).
268
269 Next, we constructed a species-specific repeat library using RepeatModeler package (v.
270 open-1.0.8; -engine ncbi -pa 35). Since the de novo identified repeats may still include parts
271 of protein-coding genes, we perform an additional filtering step and remove from the
272 database the repeats that can be found in a comprehensive set of known proteins. We
273 remove all repeats that match to a pre-computed set non-transposable proteins from our
274 protein reference database. We used custom script (filter_repeats.bash) for filtering. To
275 identify the location of repeats from this library in the genomes, we used RepeatMasker
276 (open-4.0.9).
277
278 We used the MAKER package to compute gene builds. An evidence-guided gene build was
279 computed by MAKER (-fix-nucleotides) constructing gene models from these reference
280 proteins and from the transcript sequences aligned to the genome assembly (parameters in

281 evidence_build.maker_opts.ctl). We also used prepared an ab initio build using the
282 AbInitioTraining.nf pipeline and AbInitioTraining_params.config as the parameters file.
283 Both the evidence-guided and the ab initio builds were than merged using MAKER
284 (hybrid_build_maker_opts.ctl). Finally, we performed functional annotation using the
285 FunctionalAnnotation.nf pipeline with FunctionalAnnotation_params.config as the
286 parameter file.
287
288 **Population sequencing, read mapping and variant calling**
289 We extracted DNA from the thoraces of 299 worker bees of *B. balteatus* collected from
290 across the seven sampling sites using the Qiagen Blood and Tissue kit. We prepared dual-
291 indexed libraries using the Nextera Flex kit and performed sequencing on an Illumina HiSeq
292 X to produce 2 x 150 bp reads, using an average of 36 samples per lane. We mapped reads
293 to the *B. balteatus* reference using the mem algorithm in BWA (Li & Durbin 2009). We
294 performed sorting and indexing of the resultant bam files using samtools (Li *et al.* 2009) and
295 marked duplicate reads using Picard. We used the Genome Analysis Toolkit (GATK) to call
296 variants (McKenna *et al.* 2010). We first ran HaplotypeCaller using default parameters on
297 the bam file of each sample to generate a gVCF file for each sample. We then used
298 GenomicsDBImport and GenotypeGVCFs with default parameters to call variants for all *B.*
299 *balteatus* samples together. We applied a set of hard filters using the VariantFiltration tool
300 to filter for reliable SNPs using the following thresholds: QD < 2, FS > 60, MQ < 40,
301 MQRankSum < -12.5, ReadPosRankSum < -8 (see the GATK documentation for full
302 descriptions of each filter). Only biallelic SNPs were considered for downstream analysis.
303 Samples from *B. sylvicola* and "incognitus" samples were analyzed in the same way and are
304 presented in Christmas *et al.* (2021).
305
306 **Relatedness and population structure**
307 We estimated the pairwise kinship coefficient between all samples within each species using
308 the method described in (Manichaikul *et al.* 2010) implemented in the --relatedness2 option
309 of vcftools v0.1.16 (Danecek *et al.* 2011). Worker bees from the same bumblebee colony are
310 expected to have a relatedness of 0.75 under the assumption of monandry, which is
311 reasonable for the study species (Estoup *et al.* 1995). The kinship coefficient (φ) predicted

312 from this relationship is $\frac{3}{8}$ (the probability that two alleles sampled at random from two
313 individuals are identical by descent). We estimated the lower cutoff for the inference
314 criteria to infer this level of relatedness using powers of two as described in (Manichaikul *et*
315 *al.* 2010), providing an estimate of $\frac{3}{2^{7/2}}$.
316
317 We tested for correlations between relatedness and geographic distance for each species
318 using Mantel tests with the mantel function in the 'ecodist' package in R v4.0.2. Coordinates
319 for each sample were converted to geographic distances using the R package 'geodist' and
320 these were compared to the matrix of kinship coefficients described above. We used the
321 mantel.correlog function to produce mantel correlograms and assess whether correlations
322 were significant over different geographic distances. We performed these analyses after
323 excluding sisters from the datasets to ensure any patterns we observe were not driven by
324 an effect of sampling individuals from the same colony.
325
326 We thinned the VCF files to 1 SNP every 10 kbp using the --max-missing 1 and --thin flags in
327 vcftools v.0.1.16 to ensure no missing data and to more efficiently assess population
328 structure and relatedness. We calculated p-distance matrices for *B. balteatus* samples and
329 *B. sylvicola* and "incognitus" samples combined from the thinned vcf files using VCF2Dis
330 (https://github.com/BGI-shenzhen/VCF2Dis) and then constructed neighbor-joining trees
331 using the 'ape' package in R v4.0.2. We converted the thinned vcf files to plink format and
332 performed principal components analyses using the 'adegenet' package in R v4.0.2. We
333 performed PCAs on all samples as well as after removing one of each pair of sisters
334 identified via the kinship analysis to ensure that relatedness did not heavily influence any
335 clustering. We used admixture v1.3.0 (Alexander *et al.* 2009) to assess the most likely
336 number of genetic clusters within each species. We ran admixture for K=1 through to 10,
337 with 10 iterations per K value and used the calculated cross-validation (cv) errors to assess
338 the most suitable values of K, with low cv-error indicating higher support.
339
340 **Statistical morphometric analysis**
341 We combined the phenotypic measurements made in this study with those from previous
342 collections published in (Miller-Struttmann *et al.* 2015). These measurements are ID, glossa

343 length, prementum length, and total tongue length (equal to glossa length plus prementum
344 length). This gave us three time periods to compare amongst: '2017' (samples collected for
345 this study), '2012-2014', and '1966-1980' (both sets published in (Miller-Struttmann *et al.*
346 2015)). We performed statistical analysis of variation in glossa length, prementum length,
347 and ID in R v4.0.2. We investigated correlations between all traits for each species in the
348 2017 set using the 'cor.test' function, which calculates a Pearson's product moment
349 correlation coefficient.
350
351 Within our 2017 dataset, we compared glossa lengths between the three species by fitting
352 linear models of species against glossa length with ID included as a random effect to
353 account for body size using the lm function. We then calculated least-squares mean glossa
354 lengths from this model using the lsmeans function and tested for significant differences
355 using the Tukey method for p-value adjustment for multiple testing. We employed a similar
356 approach for comparing glossa lengths between our dataset and historical datasets for each
357 species. Here we used a linear mixed effect model framework with the 'lmer' function in the
358 lme4 package in R v4.0.2, where mountain and ID were included as random effects in each
359 model. Significance of each model was assessed using analysis of variance with the 'anova'
360 function. We calculated least-squares mean glossa lengths from each model using the
361 lsmeans function and tested for significant differences using the Tukey method for p-value
362 adjustment for multiple testing.
363
364 We analyzed variation in ID between species and geographic locations for our 2017 dataset
365 using analysis of variance ('aov' function) and assessed significance of comparisons using
366 Tukey's honestly significant difference (HSD) post hoc test ('TukeyHSD' function). For
367 comparisons of ID between years, we fitted linear mixed effect models and included
368 mountain as a random effect, then compared least-squares means from the models using
369 the Tukey method for p-value adjustment for multiple testing.
370
371 **Genome-wide association study**
372 We carried out mixed linear model-based association analyses, implemented in GCTA-
373 MLMA (Yang *et al.* 2011, 2014), to identify correlations between allele frequencies and trait
374 variation across the genome for *B. balteatus* and *B. sylvicola* samples separately

375 ("incognitus" samples were left out of this analysis due to too few samples). First, we
376 created a genetic relationship matrix (GRM) from the genome wide SNPs using the --make-
377 grm function in GCTA. We removed one sample from each pair of sisters by setting a
378 relatedness cut-off of 0.67 using the '--grm-cutoff' flag. We ran the analyses for three traits
379 per species:ID, glossa length, and the residuals from a linear regression of glossa~ID to
380 control for body size. The GRM was included as a polygenic/random effect. The analysis
381 assumes phenotypes are normally distributed and so samples were removed in the tails of
382 the distributions to meet this assumption for all traits except ID in *B. sylvicola*, which was
383 normally distributed. For *B. balteatus*, this resulted in 279, 291, and 262 samples for the
384 glossa length, ID, and residuals analyses respectively. For *B. sylvicola* there were 176, 192,
385 and 152 samples for the glossa length, ID, and residuals analyses respectively. We assessed
386 divergence of p-values from neutral expectations using QQ-plots and carried out Bonferroni
387 correction of p-values using the 'p.adjust' function in R v4.0.2 to assess genome-wide
388 significance.
389
390 **Genome-wide complex trait analysis**
391 We used GCTA (Yang *et al.* 2011) to perform genome-wide complex trait analysis. We
392 estimated the phenotypic variance explained by the genome-wide SNPs using genome-
393 based restricted maximum likelihood (GREML). The analysis was performed separately for
394 each species on measures of ID, glossa length, and glossa length with ID included as a
395 covariate to control for body size. We ran each analysis twice, once using a GRM based on
396 all samples and a second time with the same GRM produced above where one from each
397 pair of sisters was removed. We calculated 95% confidence intervals around each estimate
398 by multiplying the standard error by 1.96. We used R functions provided in (Wang & Xu
399 2019) as well as the GCTA-GREML power calculator (Visscher *et al.* 2014) to perform power
400 analysis and estimate the effect sizes we can expect to detect with our population datasets.
401
402 **RESULTS**
403 **Generation of a highly-contiguous genome assembly of *B. balteatus***
404 We generated a *de novo* genome assembly for the species *B. balteatus* using Oxford
405 Nanopore (ONT) long-read and 10x Chromium linked-read sequencing and compared this to
406 the assembly for *B. sylvicola* that we recently published (Christmas *et al.* 2021). Assembly

407 statistics for BBAL_1.0 (*B. balteatus*) and BSYL_1.0 (*B. sylvicola*) are presented in table 1,
408 which includes statistics for the assemblies of *B. terrestris* (BTER_1.0) and *B. impatiens*
409 (BIMP_2.2) for comparison. Total assembly sizes of 250.1 and 252.1 Mbp for *B. balteatus*
410 and *B. sylvicola* respectively are both above the average assembly size of *Bombus* genomes
411 assembled to date of 247.9 Mbp (22 genomes, range 229.8 – 282.1 Mbp) (Sadd *et al.* 2015;
412 Heraghty *et al.* 2020; Sun *et al.* 2020). Contig N50s of 8.60 and 3.02 for BBAL_1.0 and
413 BSYL_1.0 respectively are the longest and third longest contig N50s of all published *Bombus*
414 assemblies, demonstrating these to be highly contiguous assemblies. Megabase-scale contig
415 N50 are typical of long-read assemblies, such as the ones presented here and in (Heraghty
416 *et al.* 2020), and are significantly more contiguous than those based on short-read
417 sequencing technologies such as BTER_1.0 and BIMP_2.2.
418
419 High genome completeness and accuracy is also reflected in the BUSCO analysis, with scores
420 of 99.0% (98.7% single copy, 0.3% duplicated, 0.5% fragmented, 0.5% missing) and 98.2%
421 (97.9% single copy, 0.3% duplicated, 0.4% fragmented, 1.4% missing) reported for BBAL_1.0
422 and BSYL_1.0 respectively. Whole genome alignments to the *B. terrestris* assembly (Sadd *et*
423 *al.* 2015) resulted in 93.9% and 91.1% of the *B. balteatus* and *B. sylvicola* genomes being
424 placed on 18 pseudochromosomes. Our annotation pipeline annotated 11,711 and 11,585
425 genes in BBAL_1.0 and BSYL_1.0 respectively. These are highly comparable to the 11,874
426 genes in the *B. terrestris* gene set (v. 1.0), but lower than the 12,728 genes in the *B.*
427 *impatiens* gene set (v. 2.1). Gene annotation of the *Bombus* assemblies presented in
428 (Heraghty *et al.* 2020; Sun *et al.* 2020) also resulted in a greater number of genes annotated,
429 ranging from 13,325 to 16,970 genes. BUSCO analyses of the annotated genes in our
430 assemblies do suggest that we are missing some genes from our annotations (complete
431 BUSCO genes in BBAL_01 and BSYL_01 respectively: 86.6% and 86.9%). Repeat content of
432 our assemblies is comparable to that of other *Bombus* assemblies, with 17.11% of BBAL_1.0
433 and 17.85% of BSYL_1.0 identified as repetitive by RepeatMasker (full details of repeat
434 classes in supplementary table S3). These proportions are comparable to the repeat content
435 inferred in the *B. terrestris* and *B. impatiens* genomes.
436
437 **Population resequencing indicates three species clusters, with little population structure**
438 **within species**

439 The locations of sample collections, which comprise 580 samples from across seven
440 localities in the Rocky Mountains, CO, USA, are presented in Fig. 1 (full details in table S1,
441 S2). Illumina sequencing resulted in an average genome coverage of 13.1x per sample, with
442 4.5 and 3.5 million SNPs identified in *B. balteatus* and *B. sylvicola* samples respectively
443 (supplementary table S4). These samples were initially identified morphologically as *B.*
444 *balteatus* and *B. sylvicola*, and those identified as *B. sylvicola* have been presented
445 previously (Christmas *et al.* 2021). Neighbor-joining trees produced from samples of both
446 groups of samples indicate that the *B. balteatus* samples form a single cluster whereas the
447 *B. sylvicola* samples form two clusters (Fig 1). By comparison with an additional dataset
448 (Martinet *et al.* 2019) we previously inferred the largest cluster to be comprised of *B.*
449 *sylvicola* samples whereas the second cluster is comprised of the cryptic species
450 "incognitus" (Christmas *et al.* 2021). Among samples originally designated as *B. sylvicola,*
451 24% (n=67) are identified in the separate cluster of "incognitus" whereas the remaining 76%
452 (n=214) are *B. sylvicola. Bombus balteatus* (n=299) was the most commonly observed
453 species across all localities. The proportions vary between localities (table S1). "Incognitus"
454 samples were collected on six out of the seven mountains, whereas *B. balteatus* and *B.*
455 *sylvicola* were found on all seven.
456
457 We calculated a pairwise kinship coefficient between samples to infer degrees of
458 relatedness (Manichaikul *et al.* 2010; Danecek *et al.* 2011). We inferred a set of samples that
459 were likely to be nestmates (sisters) based on them having the same maternal and paternal
460 parents, which is likely to be the case for all workers from the same colony in these species,
461 under the realistic assumption that each colony is headed by a single monandrous female
462 queen (Estoup *et al.* 1995). Those identified as sisters from the same single-mated queen
463 from the same colony were always collected foraging on the same mountain and at the
464 same or immediately neighboring site (table S1). This suggests that foraging bees were
465 generally found in the immediate vicinity of their nests. Among the 299 *B. balteatus*
466 samples, we identified 43 samples from 17 colonies that had another nestmate in the
467 dataset, indicating that we sampled a total of 273 colonies. For the 214 *B. sylvicola*, we
468 identified 14 samples from 7 colonies that met this criterion, indicating a total of 207
469 colonies. For 67 "incognitus", we identified two samples from the same colony, indicating
470 that we sampled 66 colonies.

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 “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). 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. Outlier clusters seen in the PCA plots of *B. balteatus* and “incognitus” (Fig. 2A,C, circled) are composed of highly related individuals as shown in the kinship analysis, suggesting that the effect of kinship overrides any signal of genetic structure. After removing one of each pair of sisters, nearly all *B. balteatus* samples cluster extremely tightly, with PC1 only explaining 0.8% of the variance (Fig. S1A). A similar pattern is observed for *B. sylvicola*, with PC1 explaining 1.2% of the variance (Fig. S1B). For “incognitus”, two pairs of highly related individuals are separated from the main cluster on PC1, but again only a small amount of the genetic variance is captured by PC1 (2.6%), suggesting they are still highly similar (Fig. S1C).

We assessed connectivity between colonies on different mountains by analyzing the association between kinship and geography using Mantel tests after removing sisters from the datasets (Fig. 3). We found no significant relationship between geographic distance and relatedness for *B. balteatus* (Mantel R = -0.03 (95% ci: -0.04 - -0.02), p value = 0.11; Fig. 3A,D). There were small but significant negative relationships for *B. sylvicola* (Mantel R = -0.06 (95% c.i. -0.07 - -0.04), p value = 0.0001; Fig. 3B,E) and “incognitus” (Mantel R = -0.04 (95% ci: -0.06 - -0.03), p value = 0.029; Fig. 3C,F), demonstrating greater relatedness at closer locations. In these species, Mantel correlograms indicate significant associations between kinship and geographical distance only at shorter distances (from the same or neighboring mountains), which are no longer significant at greater distances (Fig. 3E-F)). These results indicate that the limited population structure is mainly driven by the occurrence of a few highly-related samples from the same localities in the dataset.

503

504 **Decrease in tongue length is ongoing in all species**

505 We made morphological measurements for each sample (table S2). Intertegular distance
506 (ID) is considered a proxy for body size (Cane 1987). We also measured the length of two
507 parts of the tongue: glossa and prementum. These two tongue measures are correlated in
508 both species (*B. balteatus* Pearson's r = 0.45, p < 0.001; *B. sylvicola* Pearson's r = 0.38, p <
509 0.001; Figs. S2,S3). Only glossa is considered further, as it is the tongue part with most
510 relevance to feeding (Cariveau *et al.* 2016) and was measured by (Miller-Struttmann *et al.*
511 2015). Glossa is also significantly correlated with ID (*B. balteatus* Pearson's r = 0.41, p <
512 0.001; *B. sylvicola* Pearson's r = 0.26, p < 0.001) (Figs. S2,3).

513

514 A comparison of phenotypes between species shows that glossa length is substantially
515 longer in *B. balteatus*, consistent with previous knowledge (ls-means = 4.72 mm, 3.34 mm,
516 and 3.40 mm for *B. balteatus*, *B. sylvicola* and "incognitus" respectively; Tukey test, t ratio =
517 12.25 and 21.17, p < 0.001 for *B. balteatus* compared to *B. sylvicola* and "incognitus"*;* Fig.
518 4A). Average glossa length is extremely similar in *B. sylvicola* and "incognitus" (Tukey test, t
519 ratio =0.50, p = 0.87), suggesting that this character does not distinguish this newly-
520 discovered species from *B. sylvicola*. *Bombus balteatus* bees are significantly larger bees as
521 indicated by a greater ID, and *B. sylvicola* is significantly larger than "incognitus" (mean ID =
522 4.00 mm, 3.83 mm, 3.58 mm for *B. balteatus*, *B. sylvicola* and "incognitus" respectively;
523 ANOVA, F=28.75, p < 0.001, Tukey post-hoc test revealed differences are significant among
524 all species at p < 0.001; Fig. S4A).

525

526 We next compared the distribution of glossa lengths in our samples with historical data. We
527 combined *B. sylvicola* and "incognitus" for this analysis because they do not differ in glossa
528 length on average and they were not distinguished in previous studies. We find an overall
529 trend of decreasing glossa length in both *B. sylvicola*-incognitus and *B. balteatus* over time
530 (Fig. 4B,C). This was reported previously between the collections from 1966-1980 and from
531 2012-2014 for both species (Miller-Struttmann *et al.* 2015). Here we show that this trend is
532 continuing, with further reductions in glossa length between the 2012-2014 collections and
533 our collections from 2017. These reductions are significant for both *B. sylvicola*-incognitus
534 (ls-means: 2012-2014 = 3.51 mm, 2017 = 3.21 mm; Tukey test, t-ratio = 3.198, p = 0.004)

535 and *B. balteatus* (ls-means: 2012-2014 = 5.24 mm, 2017 = 4.84 mm; Tukey test, t-ratio =
536 2.79, p = 0.015). In contrast, there is no significant trend shown by ID (Fig. S4B,C), although
537 for *B. sylvicola*-incognitus*,* ID is significantly larger in both the 1966-80 and 2017 samples
538 compared to the 2012-2014 samples (ANOVA, F = 15.28, p < 0.001. Tukey post-hoc test, p <
539 0.001).
540
541 For samples collected in 2017, we compared the distribution of glossa lengths for each
542 species among the seven mountains where they were collected (Fig. S5). There is an overall
543 trend for glossa lengths to be longer in bees from Mount Evans than on other mountains in
544 all species. The distributions for *B. balteatus* and *B. sylvicola* show significant differences,
545 with samples from Mount Evans having significantly longer glossae than on Mount
546 Democrat, Horseshoe Mountain, and Pennsylvania Mountain in *B. balteatus* (ls-mean glossa
547 length on Mt. Evans = 5.34 mm, other mountains 4.51 – 4.73 mm; Tukey p < 0.01 in each
548 case; Fig. S5A) and significantly longer glossae than on all other mountains except Niwot
549 Ridge in *B. sylvicola* (ls-mean glossa length on Mt. Evans = 3.77 mm, other mountains 3.02 –
550 3.24 mm; Tukey p < 0.01 in each case; Fig. S5B). None of the trends for "incognitus" were
551 significant (Fig. S5C). Comparisons of ID between mountains revealed that *B. balteatus* bees
552 sampled on Niwot Ridge are significantly larger than those on Boreas Mountain, Mount
553 Democrat, Horseshoe Mountain, and Quail Mountain (Niwot Ridge mean ID = 4.25 mm,
554 other mountains 3.84 - 4.00 mm, ANOVA, F = 4.09, p < 0.001, Tukey post-hoc test p < 0.05 in
555 four comparisons; Fig. S6A). We did not identify any significant differences in ID of bees
556 across mountains for *B. sylvicola* (ANOVA, F = 1.83, p = 0.10) or "incognitus" (ANOVA, F =
557 1.62, p = 0.17; Fig. S6B,C).
558
559 **Genome-wide association study identifies a SNP associated with a proxy for body size**
560 We used a linear mixed-model analysis to search for correlations between allele frequencies
561 and quantitative trait variation in *B. sylvicola* and *B. balteatus.* We used the traits glossa
562 length, ID and glossa length corrected for ID (see Methods). One from each pair of sisters
563 identified in the kinship analysis was removed from all analyses to reduce the level of
564 relatedness in the sample set. We detected a genome-wide significant association between
565 ID and a SNP in intron 9 of the *CTRB1* (Chymotrypsinogen B1) gene on chromosome 10 in *B.*
566 *sylvicola* (p < 0.001 after Bonferroni correction; Fig. 5). The SNP is found at an allele

567 frequency of 0.14 across all populations, with 41 individuals heterozygous and nine
568 homozygous for the allele. The allele is present on all mountains, ranging in allele frequency
569 from 0.06 (Quail Mountain) to 0.24 (Niwot Ridge). The *CTRB1* gene is involved in production
570 of chymotrypsin, a proteolytic digestive enzyme (Giebel *et al.* 1971; Grogan & Hunt 1979;
571 Rawlings & Barrett 1994; Burgess *et al.* 1996; Matsuoka *et al.* 2015; Lazarević & Janković-
572 Tomanić 2015). Intron 9 is a large 18 Kbp intron and the significant SNP is located 2 Kbp
573 upstream of exon 10. The GWAS significance suggests it is of likely functional importance
574 and its location is suggestive of a regulatory element, such as an intron splice enhancer or
575 silencer.
576
577 Two further SNPs, located 4 bp apart on chromosome 11, also significantly associated with
578 ID ($p = 0.039$ after Bonferroni correction). However, they are not located in or close to any
579 genes according to our annotation, so any potential functional significance of these SNPs is
580 unknown. We did not find any significant associations between allele frequencies and ID in
581 *B. balteatus* (Fig. S7). Furthermore, no significant associations were found between allele
582 frequencies and glossa length, or glossa length corrected for ID in either species (Fig. S7).
583 This indicates that the trait has a polygenic or mainly environmental component. A power
584 analysis (Visscher *et al.* 2014) revealed that our datasets have sufficient power (>0.8) to
585 detect a QTL with a h^2 of ~0.1 but that loci with lower effect sizes would likely be
586 undetectable.
587
588 **Limited evidence that morphological variation has a genetic component**
589 We performed GCTA-GREML analyses (Yang *et al.* 2011) to determine the genetic
590 component of trait variation for *B. sylvicola* and *B. balteatus* (Fig. 6, supplementary table
591 S5). The "incognitus" samples were not included here due to smaller sample size. When
592 each species' entire dataset is considered, we detect a heritable component for both glossa
593 length and ID in both species, where the 95% confidence interval does not overlap zero. For
594 *B. sylvicola*, but not *B. balteatus*, this is also the case when ID is introduced as a covariate to
595 account for body size when considering glossa length. However, when we remove samples
596 that we infer to be workers from the same colony from the kinship analysis, we only detect
597 a heritable component with 95% confidence for ID in *B. balteatus* (all others then overlap
598 with zero). This indicates that a large proportion of the signal of covariance between genetic

599 and trait variance is due to the presence of sisters from the same colony. This could be a
600 confounding factor because environmental effects such as level of nutrition and colony
601 health are likely to covary with colony. However, such covariation could also reflect
602 genetics. The level of dependency of phenotype on genotype is therefore unclear from this
603 analysis.
604
605 The 95% confidence intervals for the estimate of heritability have a wide span and overlap
606 zero in many cases. The power to accurately determine heritability depends on several
607 factors, including sample size, the structure of relatedness in the samples, and the true
608 value of heritability. When samples are all completely unrelated, it has been estimated
609 that > 3,000 samples are required to achieve a SE below 0.1 (Visscher *et al.* 2014). For
610 datasets of unrelated samples of the size we have analyzed here, we estimate a power of
611 ~0.06, or ~6% probability, to detect $h^2 > 0$ for a trait with a SNP-heritability of 0.5 and a type
612 1 error rate (α) of 0.05. Greater sample size or higher levels of relatedness within the
613 dataset would therefore increase accuracy of our heritability estimates.
614
615 **DISCUSSION**
616 In this study, we generated a high-quality reference genome and performed genome
617 sequencing of a population sample of the bumblebee species *B. balteatus*. Combining this
618 data with a previous dataset from *B. sylvicola* produced the most extensive population
619 genomics dataset for bumblebees to date, with 580 samples from these two species and the
620 newly-discovered “incognitus” collected from high-altitude locations in Colorado, USA.
621 These data were combined with morphometric measurements from each sample and used
622 to investigate genetic structure, morphological evolution and the genetic basis of
623 phenotypic traits in these populations. Our main findings are 1) no evidence for gene flow
624 between species, including the recently-inferred "incognitus", but lack of population
625 structure within species, indicating high levels of connectivity among subpopulations. 2) An
626 ongoing decrease in tongue (glossa) length compared to historical measurements,
627 previously inferred to reflect the effects of decreasing floral abundance on bumble bee
628 foraging (Miller-Struttmann *et al.* 2015). 3) An association between body size and variation
629 at a single SNP located in a gene related to production of a digestive enzyme in *B. sylvicola*.
630 4) A lack of conclusive evidence for a genetic component to tongue length in both species.

631 Our analysis is unable to determine the degree of heritability underlying these traits but is
632 consistent with a highly polygenic genetic component.
633
634 This investigation adds to the number of bumblebee species with reference genomes
635 available. The genomes of the common species *B. impatiens* and *B. terrestris* were
636 sequenced using short-read technologies (Sadd *et al.* 2015). Genome assemblies are also
637 available for representatives of all 15 *Bombus* subgenera (Sun *et al.* 2020) based on short-
638 read sequencing of jumping libraries and scaffolding using Hi-C, resulting in contig N50 of
639 325 kb. In addition to the two genomes produced here, the genomes of the North American
640 species *B. terricola*, *B. bifarius*, *B. vancouverensis*, and *B. vosnesenskii* have also been
641 produced using long-read sequencing (PacBio or ONT) and have megabase-scale contig N50s
642 (Kent *et al.* 2018; Heraghty *et al.* 2020).
643
644 Identification of the previously-unknown species "incognitus" demonstrates the power of
645 population-scale genome sequencing to discover new cryptic variation (Christmas *et al.*
646 2021). None of the morphological features that we studied could distinguish between *B.*
647 *sylvicola* and "incognitus". *Bombus sylvicola* did however have a significant tendency to be
648 collected at higher altitudes and have larger body size. However, importantly for this study,
649 there were no significant differences in glossa or prementum length between the two
650 species. The wider distribution of "incognitus" is unknown, as is the true composition of
651 populations described as *B. sylvicola* in other geographical locations. Existing collections of
652 *B. sylvicola* from Colorado are likely to be a mixture of both species.
653
654 We find no evidence for population structure among samples of any of the of the three
655 species studied here. This indicates an absence of long-term geographical barriers to gene
656 flow between the high-elevation localities included here. Furthermore, although we observe
657 elevated degrees of relatedness among foragers collected on the same mountain, there is
658 no significant tendency for elevated relatedness of foragers caught on nearby mountains,
659 indicating a lack of population structure at this scale. Foragers inferred to belong to the
660 same nest (i.e. with identical parents) were always found in the same or adjacent sites,
661 consistent with the limited foraging distance (on average < 110m) inferred previously by
662 Geib et al. (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 (Lozier *et al.* 2011; Ghisbain *et al.* 2020). However, the complex topography of mountain ranges likely modulates gene flow between populations (Lozier *et al.* 2011, 2013). 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 *et al.* 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 contribute to the observed variation in body size over time as *B. sylvicola* have a significantly greater ID on average than “incognitus” (Fig. S4).

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

695 between 1966 and 2014 for *B. sylvicola* (Miller-Struttmann *et al.* 2015). According to our
696 recent measurements, the corresponding decrease in 3-5 subsequent years has been
697 12.9 %, or 3.2% per year (compared to the 2012-14 average).
698
699 Decreases in tongue-length have been previously attributed to an advantage of shorter
700 tongues for foraging on a wide range of flowers. Such generalist foraging could be favored
701 due to a general decline of floral resources observed in connection with warmer summers
702 (Miller-Struttmann *et al.* 2015). While both long- and short-tongued bees are capable of
703 accessing nectar in plants with short flower tubes, short-tongued bees are more efficient at
704 foraging from them (Inouye 1980) and are also more efficient at foraging across a diversity
705 of flower tube depths (Plowright & Plowright 1997; Arbulo *et al.* 2011; Geib & Galen 2012).
706 Short-tongued bumble bees often forage from a wider suite of plants both in these alpine
707 bumble bee species (Miller-Struttmann *et al.* 2015) and others (Heinrich 2004; Goulson &
708 Darvill 2004; Goulson *et al.* 2008b; Huang *et al.* 2015). These considerations indicate that
709 shorter tongues are likely advantageous for generalist foraging. Although short-tongued
710 bees are unable to pollinate plants with long flower tubes, they are likely important for
711 supporting a diverse array of flowering plants.
712
713 It is unclear whether the tongue-length changes we observe are the result of natural
714 selection or phenotypic plasticity. There are now many examples of rapid evolution of traits
715 driven by natural selection due to climatic events in the wild, such as a shift in beak shape
716 over a period of a few years in Darwin's finches related to competition for food during
717 drought (Lamichhaney *et al.* 2016) and genomic shifts related to cold-tolerance in the green
718 anole lizard related to severe winter storms (Campbell-Staton *et al.* 2017). Human activity
719 may also promote rapid adaptation by natural selection, as demonstrated by the evolution
720 of longer beaks in great tits promoted by the use of bird feeders in the UK (Bosse *et al.*
721 2017). Global climate change is also expected to be a driver of rapid evolution, but it is often
722 difficult to establish whether trends of phenotypic change result from evolution or
723 phenotypic plasticity (Merilä & Hendry 2014). Long-term changes in breeding timing in great
724 tits (Charmantier *et al.* 2008) and body mass in red-billed gulls (Teplitsky *et al.* 2008) that
725 track temperature increases have been shown to result solely from phenotypic plasticity.
726

727 To address the causes of the changes in bumblebee tongue length observed here, we
728 attempted to uncover the genetic basis of morphological variation using genome-wide
729 association studies (GWAS) and genome-wide complex trait analysis (GCTA). No significant
730 associations were observed for tongue length in the genome of any of the species using
731 GWAS. However, a significant association with body size was identified in *B. sylvicola* at a
732 single SNP in the *CTRB1* (Chymotrypsinogen B1) gene. The lack of association at neighboring
733 SNPs could be attributable to high rates of meiotic recombination and low levels of linkage
734 disequilibrium in bumblebees (Kawakami *et al.* 2019). The *CTRB1* gene produces a precursor
735 of chymotrypsin, a key digestive enzyme in animals, which is likely important for pollen
736 digestion in bees (Giebel *et al.* 1971; Grogan & Hunt 1979; Rawlings & Barrett 1994; Burgess
737 *et al.* 1996; Matsuoka *et al.* 2015; Lazarević & Janković-Tomanić 2015). The associated SNP
738 is found in a large intron (18 Kbp) of this gene, 2 Kbp upstream of the nearest exon. We lack
739 any functional annotation for this SNP but its location and GWAS significance suggests it
740 may play a role in gene regulation. For example, if located within an intronic splicing
741 enhancer or silencer, it may result in variation in expression levels of different isoforms of
742 the CTRB1 enzyme being produced. Such changes could influence body size through an
743 effect on the efficiency of digestion.
744
745 We detected a significant signal of covariance of trait measurements with relatedness for
746 both tongue length and ID using GCTA when closely-related individuals were included in the
747 dataset. However, no significant signal of heritability in morphological traits was detected
748 when the dataset was purged of close relatives. The first result could potentially reflect a
749 genetic component, but it is not possible to disentangle this component from the shared
750 environmental effects due to close relatives sharing a nest. This analysis is therefore
751 underpowered to detect genetic effects due to low sample size and a general lack of related
752 individuals in the dataset. A possible explanation for our results is that the morphological
753 traits tongue length and ID have a highly polygenic genetic component, with multiple QTL of
754 small effect ($h^2 < 0.1$), such that GWAS and GCTA were underpowered to detect QTL and
755 estimate heritability. The size of the genetic component of variation in both traits is
756 therefore still unknown in each species.
757

It therefore remains unclear whether the observed changes in tongue length are caused by natural selection acting on functional genetic variation, or whether they are the result of phenotypic plasticity. There is large variation in body size between individuals in bumblebee nests, indicating substantial phenotypic plasticity in this trait (Peat *et al.* 2005). This variability could be explained by a correlation between larval position in the nest and size, which is related to feeding intensity (Couvillon & Dornhaus 2009). There is evidence from solitary bees of an effect of temperature on body size (Scaven & Rafferty 2013), although such an effect is likely to be less pronounced in social insects such as bumblebees that regulate the temperature of their nests. However, it is unclear whether tongue length also exhibits plasticity, as this trait correlates with body size among nestmates (Peat *et al.* 2005), indicating that plasticity in tongue length independent of body size is less pronounced. However, our observation of significant differences in mean tongue length between mountains could potentially reflect phenotypic plasticity.

In summary, we have shown that decreasing tongue length observed over the last six decades is still ongoing in populations of the bumblebee species *B. sylvicola* and *B. balteatus* (and the recently-reported "incognitus") at high-altitudes in Colorado. We have generated the genomic resources to study these species on the genetic level in the form of highly-contiguous genome assemblies and comprehensively characterized genome variation. Our results indicate that the complex topology of the mountain landscape in Colorado does not contain significant barriers to mating and dispersal of bumblebees between within this area. We are unable to conclude whether the observed morphological shifts over time are the result of 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

789 All sequence data presented here are available at NCBI under BioProject IDs PRJNA646847
790 and PRJNA704506. Scripts and annotation parameters used in the study are available at
791 GitHub (https://github.com/matt-webster-lab/alpine_bumblebees). VCF files containing
792 genotypic variation are available at the Dryad Digital Repository
793 (https://doi.org/10.5061/dryad.mgqnk990p).
794

795 **ACKNOWLEDGEMENTS**

796 This research was funded by Swedish Research Council Formas grant 2016-00535 and a
797 SciLifeLab National Biodiversity Project NP00046 to MTW. MK is financially supported by the
798 Knut and Alice Wallenberg Foundation as part of the National Bioinformatics Infrastructure
799 Sweden at SciLifeLab. The authors acknowledge support from the National Genomics
800 Infrastructure in Stockholm and Uppsala funded by Science for Life Laboratory, the Knut and
801 Alice Wallenberg Foundation and the Swedish Research Council. The computations and data
802 handling were enabled by resources provided by the Swedish National Infrastructure for
803 Computing (SNIC) at UPPMAX partially funded by the Swedish Research Council through
804 grant agreement no. 2018-05973. We thank the U.S. Forest Service, Dr. Rosemond Greinetz
805 and the Mountain Area Land Trust for providing access to field sites. We thank Jacqueline
806 Staab and Michael Osbourn who aided in specimen collection, identification and
807 preservation and Lisa Danback, Kate Schleicher, Ying Thao, Anna Grobelny and Breana Cook
808 who carried out phenotypic measurements.
809

810 **REFERENCES**

811 Alexander DH, Novembre J, Lange K (2009) Fast model-based estimation of ancestry in
812 unrelated individuals. *Genome Research*, **19**, 1655–1664.
813 Arbulo N, Santos E, Salvarrey S, Invernizzi C (2011) Proboscis Length and Resource
814 Utilization in Two Uruguayan Bumblebees: Bombus atratus Franklin and Bombus
815 bellicosus Smith (Hymenoptera: Apidae). *Neotropical Entomology*, **40**, 72–77.
816 Bosse M, Spurgin LG, Laine VN, Cole EF, Firth JA, Gienapp P, … Slate J (2017) Recent natural
817 selection causes adaptive evolution of an avian polygenic trait. *Science*, **358**, 365–
818 368.
819 Burgess EPJ, Malone LA, Christeller JT (1996) Effects of two proteinase inhibitors on the
820 digestive enzymes and survival of honey bees (Apis mellifera). *Journal of Insect*
821 *Physiology*, **42**, 823–828.
822 Cameron SA, Lozier JD, Strange JP, Koch JB, Cordes N, Solter LF, Griswold TL (2011) Patterns
823 of widespread decline in North American bumble bees. *Proceedings of the National*
824 *Academy of Sciences*, **108**, 662–667.

825 Cameron SA, Sadd BM (2020) Global Trends in Bumble Bee Health. *Annual Review of*
826 *Entomology*, **65**, 209–232.
827 Campbell-Staton SC, Cheviron ZA, Rochette N, Catchen J, Losos JB, Edwards SV (2017)
828 Winter storms drive rapid phenotypic, regulatory, and genomic shifts in the green
829 anole lizard. *Science*, **357**, 495–498.
830 Cane JH (1987) Estimation of Bee Size Using Intertegular Span (Apoidea). *Journal of the*
831 *Kansas Entomological Society*, **60**, 145–147.
832 Cariveau DP, Nayak GK, Bartomeus I, Zientek J, Ascher JS, Gibbs J, Winfree R (2016) The
833 Allometry of Bee Proboscis Length and Its Uses in Ecology. *PLOS ONE*, **11**, e0151482.
834 Charmantier A, McCleery RH, Cole LR, Perrins C, Kruuk LEB, Sheldon BC (2008) Adaptive
835 Phenotypic Plasticity in Response to Climate Change in a Wild Bird Population.
836 *Science*, **320**, 800–803.
837 Christmas MJ, Jones JC, Olsson A, Wallerman O, Bunikis I, Kierczak M, … Webster MT (2021)
838 Genetic Barriers to Historical Gene Flow between Cryptic Species of Alpine
839 Bumblebees Revealed by Comparative Population Genomics. *Molecular Biology and*
840 *Evolution*.
841 Couvillon MJ, Dornhaus A (2009) Location, location, location: larvae position inside the nest
842 is correlated with adult body size in worker bumble-bees (Bombus impatiens).
843 *Proceedings of the Royal Society B: Biological Sciences*, **276**, 2411–2418.
844 Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, … Durbin R (2011) The
845 variant call format and VCFtools. *Bioinformatics*, **27**, 2156–2158.
846 Darvill B, Ellis JS, Lye GC, Goulson D (2006) Population structure and inbreeding in a rare and
847 declining bumblebee, Bombus muscorum (Hymenoptera: Apidae). *Molecular*
848 *Ecology*, **15**, 601–611.
849 Darvill B, Knight ME, Goulson D (2004) Use of Genetic Markers to Quantify Bumblebee
850 Foraging Range and Nest Density. *Oikos*, **107**, 471–478.
851 Ellis JS, Knight ME, Darvill B, Goulson D (2006) Extremely low effective population sizes,
852 genetic structuring and reduced genetic diversity in a threatened bumblebee
853 species, Bombus sylvarum (Hymenoptera: Apidae). *Molecular Ecology*, **15**, 4375–
854 4386.
855 Elsen PR, Tingley MW (2015) Global mountain topography and the fate of montane species
856 under climate change. *Nature Climate Change*, **5**, 772–776.
857 Estoup A, Scholl A, Pouvreau A, Solignac M (1995) Monoandry and polyandry in bumble
858 bees (Hymenoptera; Bombinae) as evidenced by highly variable microsatellites.
859 *Molecular Ecology*, **4**, 89–93.
860 Geib JC, Galen C (2012) Tracing impacts of partner abundance in facultative pollination
861 mutualisms: from individuals to populations. *Ecology*, **93**, 1581–1592.
862 Geib JC, Strange JP, Galen C (2015) Bumble bee nest abundance, foraging distance, and
863 host-plant reproduction: implications for management and conservation. *Ecological*
864 *Applications*, **25**, 768–778.
865 Ghisbain G, Lozier JD, Rahman SR, Ezray BD, Tian L, Ulmer JM, … Hines HM (2020)
866 Substantial genetic divergence and lack of recent gene flow support cryptic
867 speciation in a colour polymorphic bumble bee (Bombus bifarius) species complex.
868 *Systematic Entomology*, **45**, 635–652.
869 Giebel W, Zwilling R, Pfleiderer G (1971) The evolution of endopeptidases—XII. The
870 proteolytic enzymes of the honeybee (Apis Mellifica L.): Purification and
871 characterization of endopeptidases in the midgut of adult workers and comparative

studies on the endopeptidase-pattern of different castes and on different ontogenetic stages. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **38**, 197–210.

Gienapp P, Fior S, Guillaume F, Lasky JR, Sork VL, Csilléry K (2017) Genomic Quantitative Genetics to Study Evolution in the Wild. *Trends in Ecology & Evolution*, **32**, 897–908.

Goulson D (2003) *Bumblebees: Their Behaviour and Ecology*. Oxford University Press.

Goulson D, Darvill B (2004) Niche overlap and diet breadth in bumblebees; are rare species more specialized in their choice of flowers? *Apidologie*, **35**, 55–63.

Goulson D, Hanley ME, Darvill B, Ellis JS, Knight ME (2005) Causes of rarity in bumblebees. *Biological Conservation*, **122**, 1–8.

Goulson D, Lye GC, Darvill B (2008a) Decline and conservation of bumble bees. *Annual Review of Entomology*, **53**, 191–208.

Goulson D, Lye GC, Darvill B (2008b) Diet breadth, coexistence and rarity in bumblebees. *Biodiversity and Conservation*, **17**, 3269–3288.

Goulson D, Nicholls E, Botías C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science (New York, N.Y.)*, **347**, 1255957.

Grabherr MG, Russell P, Meyer M, Mauceli E, Alföldi J, Di Palma F, Lindblad-Toh K (2010) Genome-wide synteny through highly sensitive sequence alignment: Satsuma. *Bioinformatics*, **26**, 1145–1151.

Grogan DE, Hunt JH (1979) Pollen proteases: Their potential role in insect digestion. *Insect Biochemistry*, **9**, 309–313.

Heinrich B (2004) *Bumblebee Economics*. Harvard University Press, Cambridge.

Heraghty SD, Sutton JM, Pimsler ML, Fierst JL, Strange JP, Lozier JD (2020) De Novo Genome Assemblies for Three North American Bumble Bee Species: Bombus bifarius, Bombus vancouverensis, and Bombus vosnesenskii. *G3: Genes, Genomes, Genetics*, **10**, 2585–2592.

Huang J, An J, Wu J, Williams PH (2015) Extreme Food-Plant Specialisation in Megabombus Bumblebees as a Product of Long Tongues Combined with Short Nesting Seasons. *PLOS ONE*, **10**, e0132358.

Inouye DW (1980) The effect of proboscis and corolla tube lengths on patterns and rates of flower visitation by bumblebees. *Oecologia*, **45**, 197–201.

Jackson JM, Pimsler ML, Oyen KJ, Koch-Uhuad JB, Herndon JD, Strange JP, … Lozier JD (2018) Distance, elevation and environment as drivers of diversity and divergence in bumble bees across latitude and altitude. *Molecular Ecology*, **27**, 2926–2942.

Jha S (2015) Contemporary human-altered landscapes and oceanic barriers reduce bumble bee gene flow. *Molecular Ecology*, **24**, 993–1006.

Jha S, Kremen C (2013) Resource diversity and landscape-level homogeneity drive native bee foraging. *Proceedings of the National Academy of Sciences*, **110**, 555–558.

Kawakami T, Wallberg A, Olsson A, Wintermantel D, Miranda JR de, Allsopp M, … Webster MT (2019) Substantial Heritable Variation in Recombination Rate on Multiple Scales in Honeybees and Bumblebees. *Genetics*, genetics.302008.2019.

Kearns CA, Inouye DW (1993) *Techniques for Pollination Biologists*. University Press of Colorado, Boulder.

Kent CF, Dey A, Patel H, Tsvetkov N, Tiwari T, MacPhail VJ, … Zayed A (2018) Conservation Genomics of the Declining North American Bumblebee Bombus terricola Reveals Inbreeding and Selection on Immune Genes. *Frontiers in Genetics*, **9**.

Kerr JT, Pindar A, Galpern P, Packer L, Potts SG, Roberts SM, … Pantoja A (2015) CLIMATE CHANGE. Climate change impacts on bumblebees converge across continents. *Science (New York, N.Y.)*, **349**, 177–180.

Knight ME, Martin AP, Bishop S, Osborne JL, Hale RJ, Sanderson RA, Goulson D (2005) An interspecific comparison of foraging range and nest density of four bumblebee (Bombus) species. *Molecular Ecology*, **14**, 1811–1820.

Koch JB, Looney C, Sheppard WS, Strange JP (2017) Patterns of population genetic structure and diversity across bumble bee communities in the Pacific Northwest. *Conservation Genetics*, **18**, 507–520.

Koch J, Strange J, Williams P (2014) *Bumble Bees of the Western United States*. U.S. Forest Service.

Lamichhaney S, Han F, Berglund J, Wang C, Almen MS, Webster MT, … Andersson L (2016) A beak size locus in Darwins finches facilitated character displacement during a drought. *Science*, **352**, 470–474.

Lazarević J, Janković-Tomanić M (2015) Dietary and phylogenetic correlates of digestive trypsin activity in insect pests. *Entomologia Experimentalis et Applicata*, **157**, 123–151.

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, **25**, 1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, … Durbin R (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, **25**, 2078–2079.

Lozier JD, Strange JP, Koch JB (2013) Landscape heterogeneity predicts gene flow in a widespread polymorphic bumble bee, Bombus bifarius (Hymenoptera: Apidae). *Conservation Genetics*, **14**, 1099–1110.

Lozier JD, Strange JP, Stewart IJ, Cameron SA (2011) Patterns of range-wide genetic variation in six North American bumble bee (Apidae: Bombus) species. *Molecular Ecology*, **20**, 4870–4888.

Manichaikul A, Mychaleckyj JC, Rich SS, Daly K, Sale M, Chen W-M (2010) Robust relationship inference in genome-wide association studies. *Bioinformatics*, **26**, 2867–2873.

Marshall L, Perdijk F, Dendoncker N, Kunin W, Roberts S, Biesmeijer JC (2020) Bumblebees moving up: shifts in elevation ranges in the Pyrenees over 115 years. *Proceedings. Biological Sciences*, **287**, 20202201.

Martinet B, Lecocq T, Brasero N, Gerard M, Urbanová K, Valterová I, … Rasmont P (2019) Integrative taxonomy of an arctic bumblebee species complex highlights a new cryptic species (Apidae: Bombus). *Zoological Journal of the Linnean Society*, **187**, 599–621.

Matsuoka T, Takasaki A, Mishima T, Kawashima T, Kanamaru Y, Nakamura T, Yabe T (2015) Expression and characterization of honeybee, Apis mellifera, larva chymotrypsin-like protease. *Apidologie*, **46**, 167–176.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, … DePristo MA (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome research*, **20**, 1297–1303.

Merilä J, Hendry AP (2014) Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. *Evolutionary Applications*, **7**, 1–14.

964 Miller-Struttmann NE, Geib JC, Franklin JD, Kevan PG, Holdo RM, Ebert-May D, … Galen C
965 (2015) Functional mismatch in a bumble bee pollination mutualism under climate
966 change. *Science*, **349**, 1541–1544.
967 Pauls SU, Nowak C, Bálint M, Pfenninger M (2013) The impact of global climate change on
968 genetic diversity within populations and species. *Molecular Ecology*, **22**, 925–946.
969 Peat J, Tucker J, Goulson D (2005) Does intraspecific size variation in bumblebees allow
970 colonies to efficiently exploit different flowers? *Ecological Entomology*, **30**, 176–181.
971 Plowright CMS, Plowright RC (1997) THE ADVANTAGE OF SHORT TONGUES IN BUMBLE BEES
972 (BOMBUS) — ANALYSES OF SPECIES DISTRIBUTIONS ACCORDING TO FLOWER
973 COROLLA DEPTH, AND OF WORKING SPEEDS ON WHITE CLOVER. *The Canadian*
974 *Entomologist*, **129**, 51–59.
975 Rawlings ND, Barrett AJ (1994) Families of serine peptidases. *Methods in Enzymology*, **244**,
976 19–61.
977 Razgour O, Forester B, Taggart JB, Bekaert M, Juste J, Ibáñez C, … Manel S (2019)
978 Considering adaptive genetic variation in climate change vulnerability assessment
979 reduces species range loss projections. *Proceedings of the National Academy of*
980 *Sciences*, **116**, 10418–10423.
981 Ruan J, Li H (2020) Fast and accurate long-read assembly with wtdbg2. *Nature Methods*, **17**,
982 155–158.
983 Sadd BM, Barribeau SM, Bloch G, Graaf DC de, Dearden P, Elsik CG, … Worley KC (2015) The
984 genomes of two key bumblebee species with primitive eusocial organization.
985 *Genome Biology*, **16**, 76.
986 Santure AW, Garant D (2018) Wild GWAS—association mapping in natural populations.
987 *Molecular Ecology Resources*, **18**, 729–738.
988 Scaven VL, Rafferty NE (2013) Physiological effects of climate warming on flowering plants
989 and insect pollinators and potential consequences for their interactions. *Current*
990 *Zoology*, **59**, 418–426.
991 Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image
992 analysis. *Nature Methods*, **9**, 671–675.
993 Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM (2015) BUSCO:
994 assessing genome assembly and annotation completeness with single-copy
995 orthologs. *Bioinformatics*, **31**, 3210–3212.
996 Soroye P, Newbold T, Kerr J (2020) Climate change contributes to widespread declines
997 among bumble bees across continents. *Science*, **367**, 685–688.
998 Sun C, Huang J, Wang Y, Zhao X, Su L, Thomas GWC, … Mueller RL (2020) Genus-wide
999 characterization of bumblebee genomes provides insights into their evolution and
1000 variation in ecological and behavioral traits. *Molecular Biology and Evolution*.
1001 Teplitsky C, Mills JA, Alho JS, Yarrall JW, Merilä J (2008) Bergmann's rule and climate change
1002 revisited: Disentangling environmental and genetic responses in a wild bird
1003 population. *Proceedings of the National Academy of Sciences*, **105**, 13492–13496.
1004 Visscher PM, Hemani G, Vinkhuyzen AAE, Chen G-B, Lee SH, Wray NR, … Yang J (2014)
1005 Statistical Power to Detect Genetic (Co)Variance of Complex Traits Using SNP Data in
1006 Unrelated Samples. *PLOS Genetics*, **10**, e1004269.
1007 Wang M, Xu S (2019) Statistical power in genome-wide association studies and quantitative
1008 trait locus mapping. *Heredity*, **123**, 287–306.

Whitley KM (2018) Genetic structure and gene flow barriers among populations of an alpine bumble bee (Bombus balteatus) in the central Rocky Mountains. M.Sc. Thesis. Appalachian State University.

Williams PH, Berezin MV, Cannings SG, Cederberg B, Ødegaard F, Rasmussen C, … Byvaltsev AM (2019) The arctic and alpine bumblebees of the subgenus Alpinobombus revised from integrative assessment of species' gene coalescents and morphology (Hymenoptera, Apidae, Bombus). *Zootaxa*, **4625**, zootaxa.4625.1.1.

Williams P, Thorp R, Richardson L, Colla (2014) *Bumble Bees of North America*.

Woodard SH, Lozier JD, Goulson D, Williams PH, Strange JP, Jha S (2015) Molecular tools and bumble bees: revealing hidden details of ecology and evolution in a model system. *Molecular Ecology*, **24**, 2916–2936.

Yang J, Lee SH, Goddard ME, Visscher PM (2011) GCTA: A Tool for Genome-wide Complex Trait Analysis. *The American Journal of Human Genetics*, **88**, 76–82.

Yang J, Zaitlen NA, Goddard ME, Visscher PM, Price AL (2014) Advantages and pitfalls in the application of mixed-model association methods. *Nature Genetics*, **46**, 100–106.

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.

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

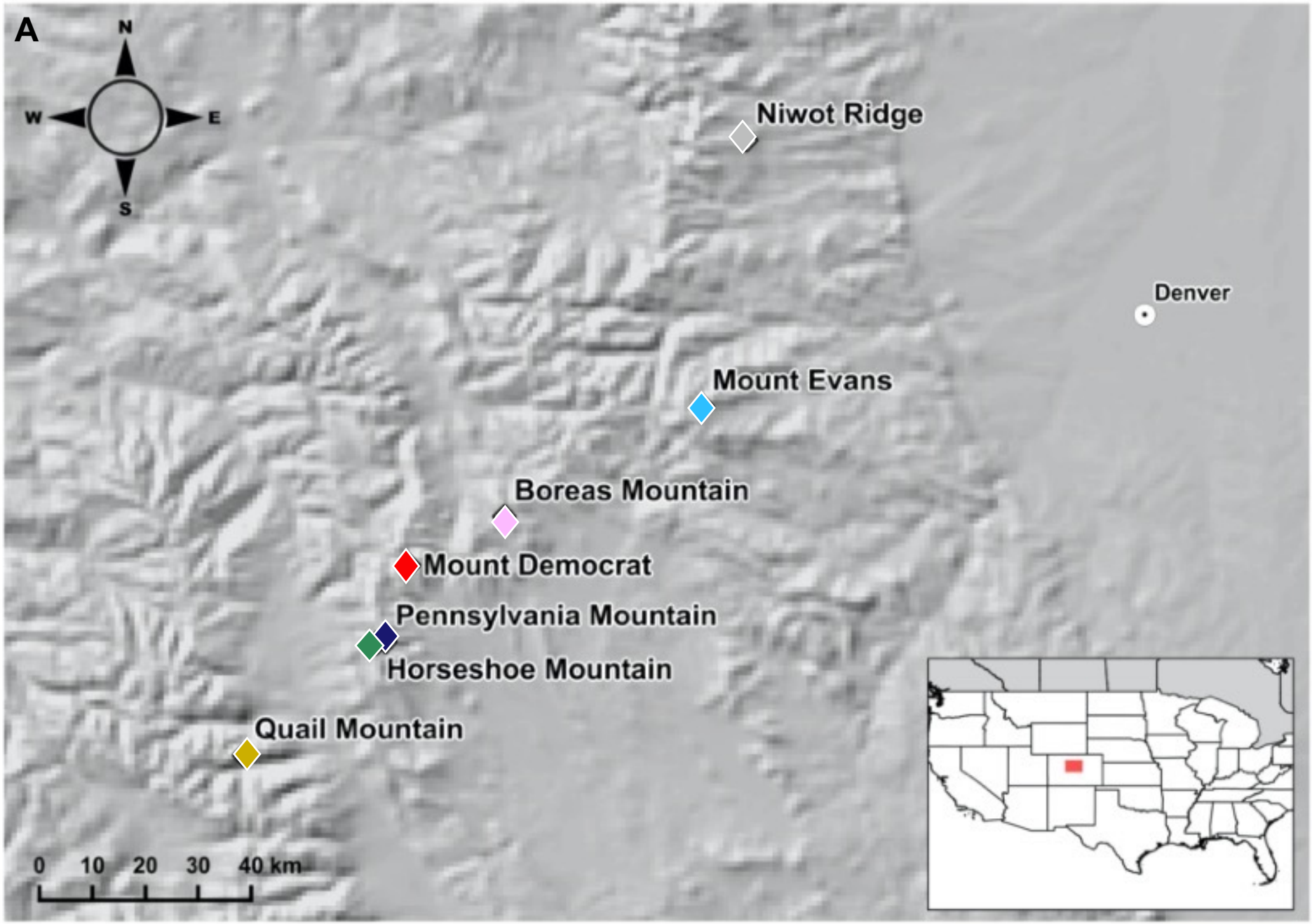
1056 lengths, correcting for body size and sample location, per sampling period for (B) *B.*
1057 *balteatus* and (C) *B. sylvicola* and "incognitus" combined. In all plots, error bars represent ls-
1058 means ± standard error and significant differences between classes are indicated by
1059 asterisks ($p < 0.05$).
1060
1061 **Figure 5. Genome-wide association Manhattan plot.** (A) The $-\log_{10}$ p-values from an
1062 association test using a mixed linear model between allele frequencies and intertegular
1063 distance (ID) are plotted for 2,705,361 genome-wide SNPs against their genome positions
1064 over the 18 *Bombus balteatus* pseudochromosomes. Red dashed line indicates genome-
1065 wide significance after Bonferroni correction for $p = 0.01$. A single SNP on chromosome 10,
1066 coloured and circled in red, had a $-\log_{10}$ p-value above this threshold. Green dashed line
1067 indicates genome-wide significance after Bonferroni correction for $p = 0.05$. Two
1068 neighbouring SNPs located on chromosome 14, coloured green, had $-\log_{10}$ p-values above
1069 this threshold. (B) Zoomed-in plot of the significant SNP in chromosome 10 showing its
1070 location within an intron of the *CTRB1* gene. Exons of this gene are represented by purple
1071 boxes, introns are represented by dashed purple lines.
1072
1073 **Figure 6. Estimates of SNP-heritability for two traits in *Bombus* species.** The proportion of
1074 variance in glossa length and intertegular distance (ID) explained by all genome-wide SNPs
1075 (the SNP-based heritability) was calculated using the genome-base restricted maximum
1076 likelihood (GREML) method in GCTA. These were calculated separately for *B. sylvicola* (left
1077 panel) and *B. balteatus* (right panel). We included ID as a covariate with glossa length to
1078 control for body size. Error bars indicate 95% confidence intervals around estimates. Each
1079 analysis was carried out twice, once with all samples (red), and a second time with one from
1080 each pair of sisters removed (black), to assess the effect of including highly related samples
1081 in the analysis.
1082

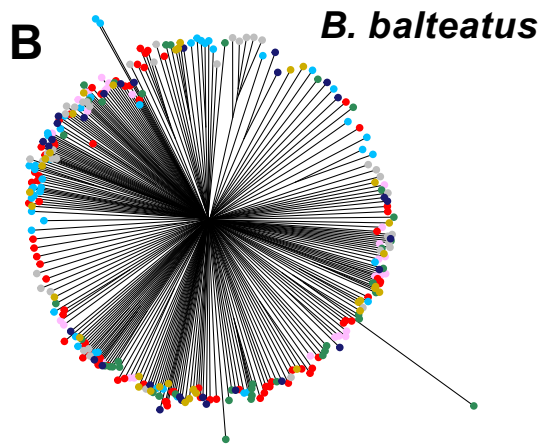
Table 1: Assembly metrics for the two genomes, with two other published *Bombus* genomes included for comparison

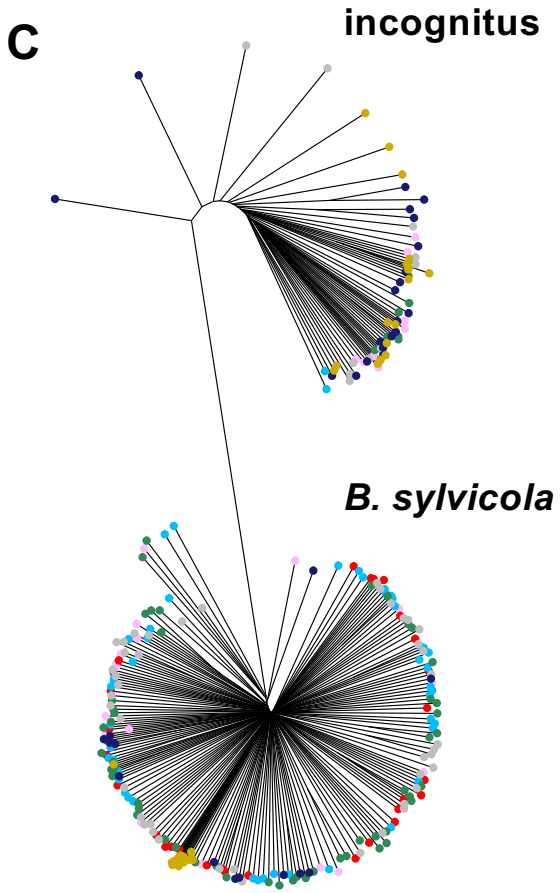
Species	*B. balteatus*	*B. sylvicola*	*B. terrestris*	*B. impatiens*
Assembly	BBAL_1.0	BSYL_1.0	Bter_1.0	BIMP_2.2
Size (Mbp)†	250.07	252.08	248.65 (236.38)	246.86 (241.98)
Scaffolds (n)	-	-	5,678	5,460
Contigs (n)	336	592	10,672	16,060
Contig N50 (Mbp)	8.60	3.02	0.08	0.06
Contig L50 (n)	12	28	890	54
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

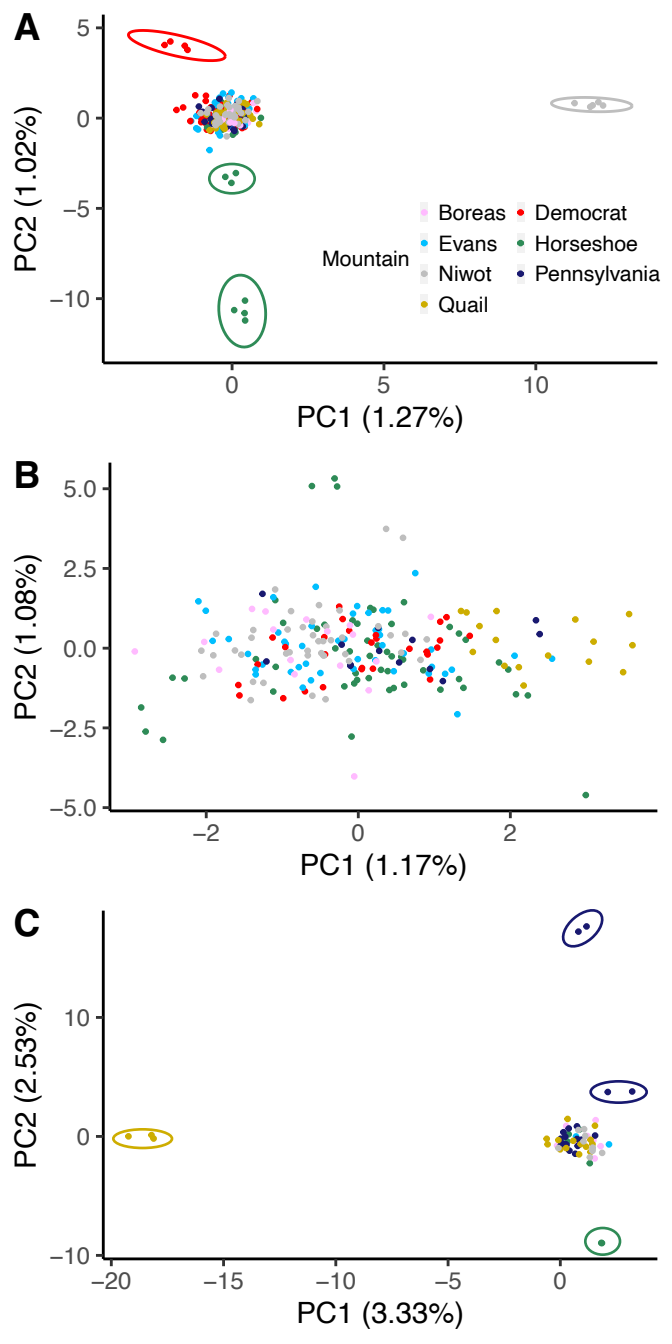
*BUSCO analysis using the OrthoDB v. 10, Hymenoptera dataset.

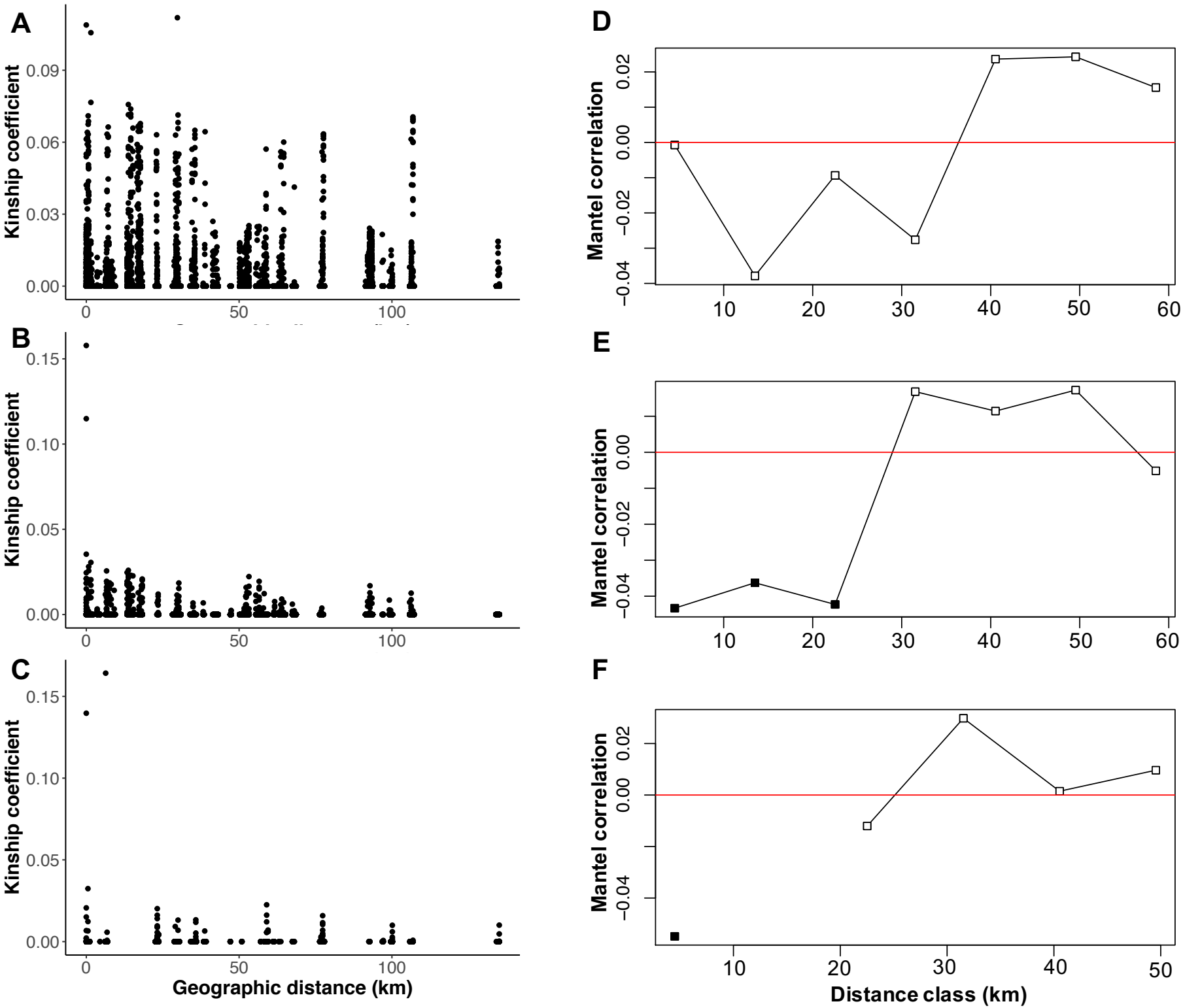
† Numbers in brackets represent total ungapped length

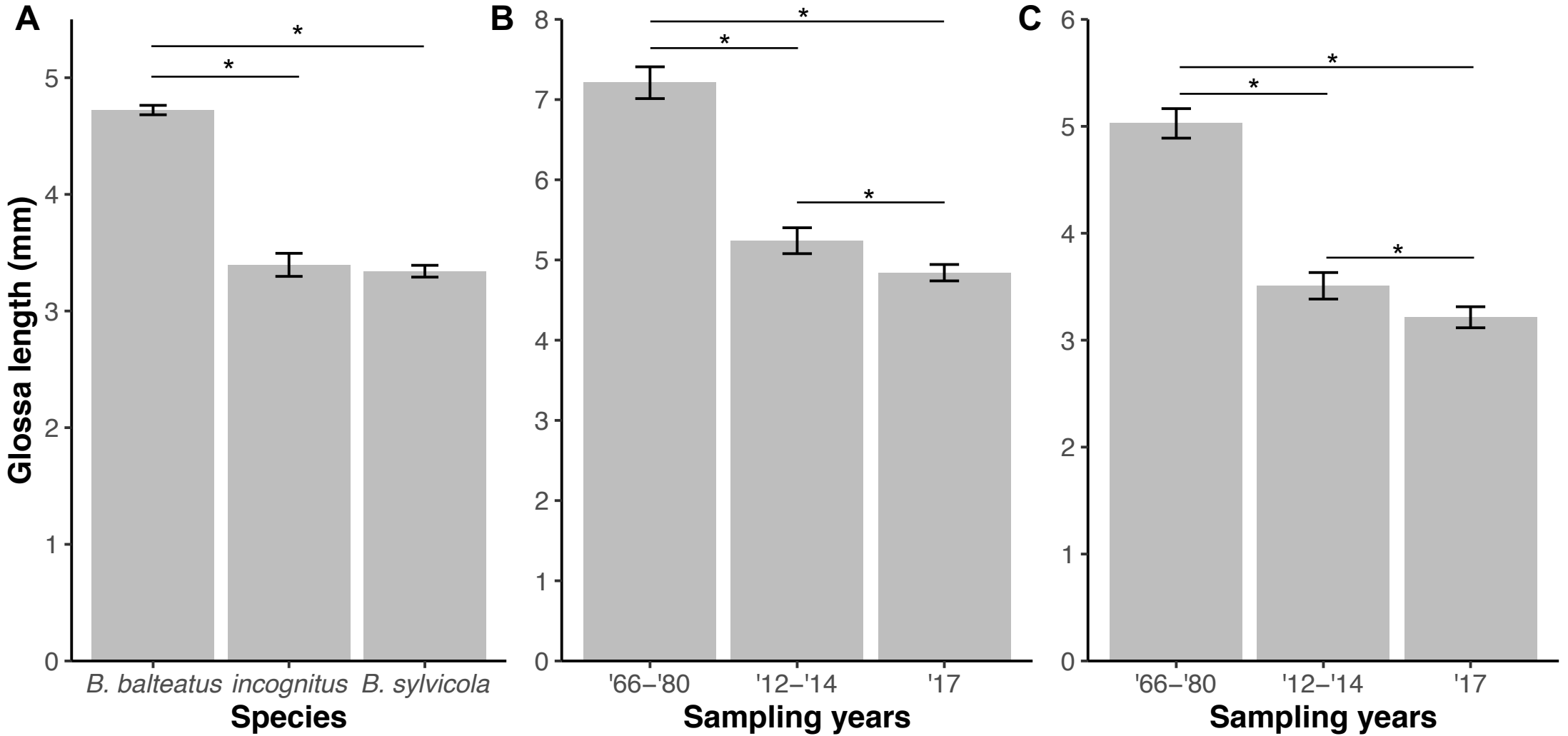

A
N
W
E
S
Niwot Ridge
Denver
Mount Evans
Boreas Mountain
Mount Democrat
Pennsylvania Mountain
Horseshoe Mountain
Quail Mountain
0 10 20 30 40 km

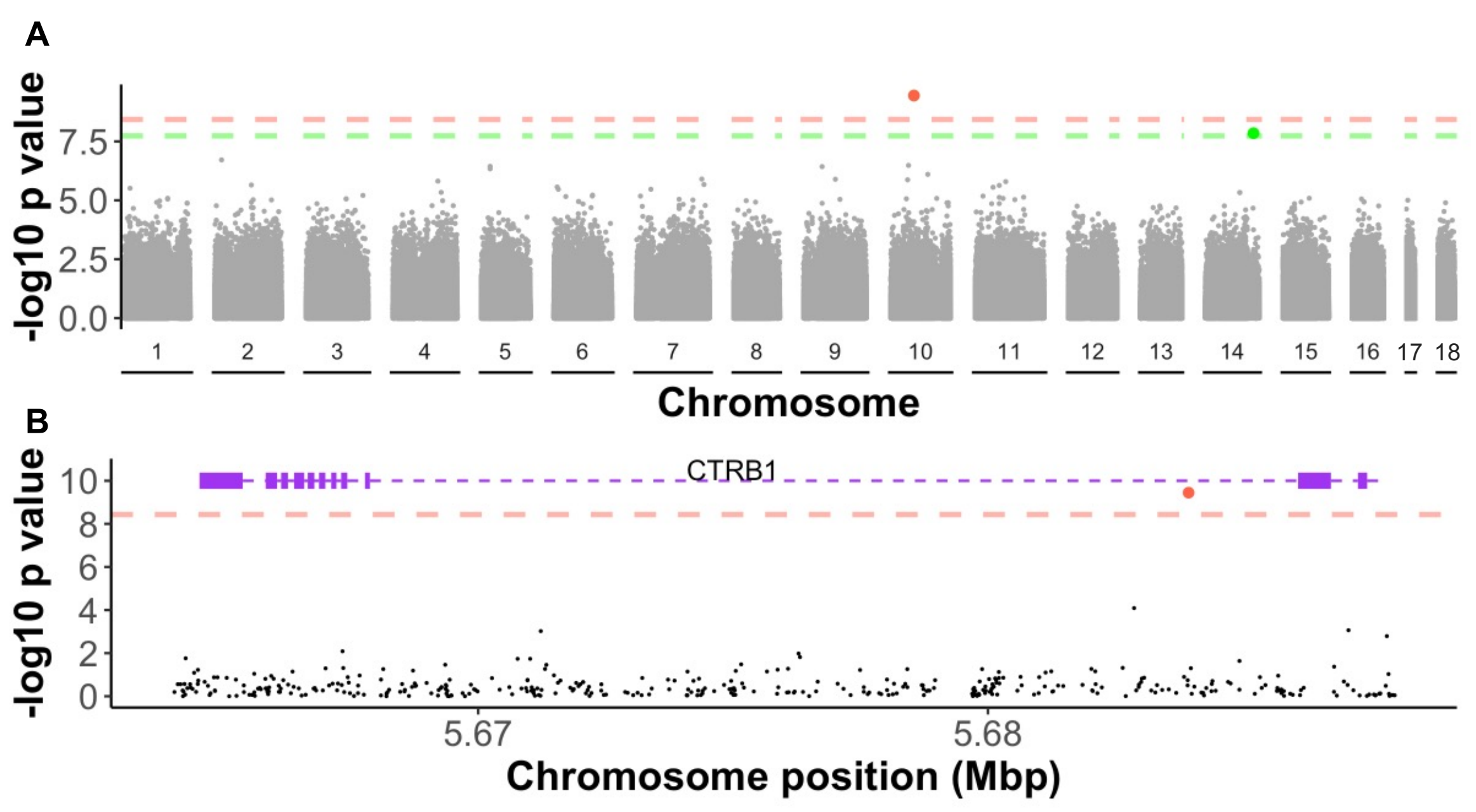

B
B. balteatus

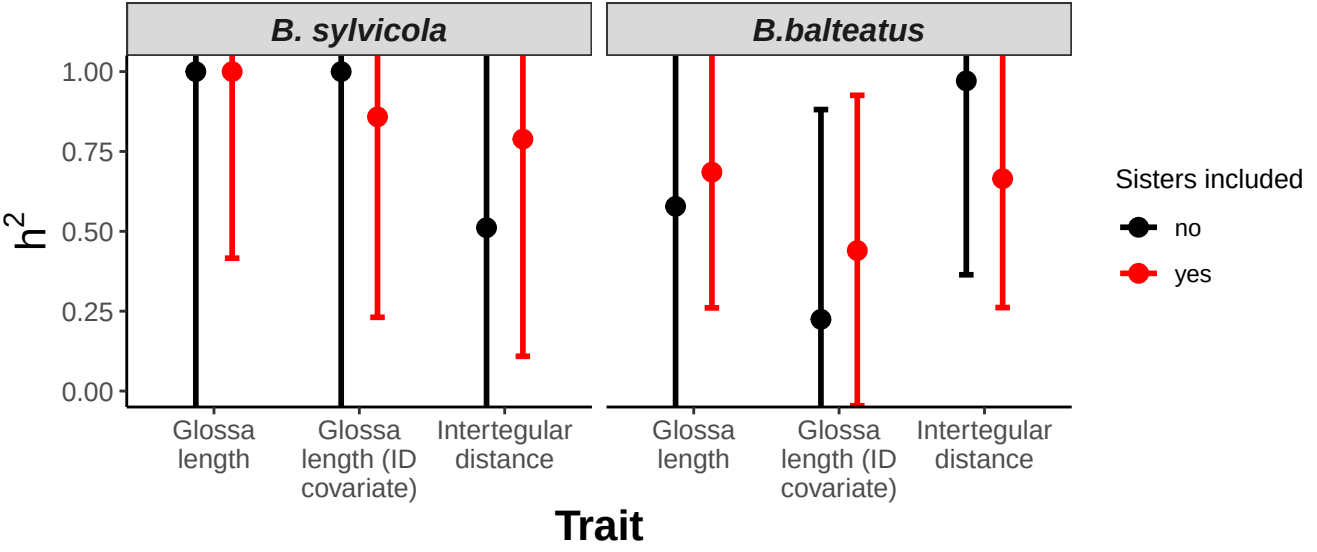

C
incognitus
B. sylvicola


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%)


A
B
C
D
E
F
Kinship coefficient
Mantel correlation
Geographic distance (km)
Distance class (km)
0.00
0.03
0.06
0.09
0.05
0.10
0.15
0
50
100
−0.04
−0.02
0.00
0.02
10
20
30
40
50
60


A
B
C
Glossa length (mm)
0
1
2
3
4
5
6
7
8
*
B. balteatus
incognitus
B. sylvicola
Species
'66–'80
'12–'14
'17
Sampling years


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)


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