

¹ Metagenomic Barcoding of Pollen Loads Offers Insights on the
² Foraging Patterns of Queen Bumble Bees

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⁴ **Abstract**

- 5 1) DNA Barcoding has been successful for the rapid analysis of ecological assemblages. Although
- 6 barcoding in the plant kingdom barcoding has been more difficult than others, and hence may begin
- 7 to lag behind other kingdoms.
- 8 2) Here we test the utilization of Angiosperms 353 probes to barcode plant species found in pollen
- 9 loads collected from Queen Bumble Bees.
- 10 3) To verify the accuracy for this barcoding system we compared the data to museum species, obser-
- 11 vation studies, and species distribution modelling to identify likely candidate species.
- 12 4) By utilizing Species distribution modelling we allow users to create a regionally appropriate sequence
- 13 databases which may use increase the alignment algorithms minimizing need for large computational
- 14 power, and run time.
- 15 5) We show that the Angiosperms 353 probes, which are currently being used in the largest ever plant
- 16 systematic endeavor, offers significant promise to metagenomic approaches.
- 17 6) Understanding plants in ecological contexts and understandings of their synecology.

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¹⁸ 1 | INTRODUCTION

¹⁹ The inability to reliably identify plants to terminal taxon can limit our understanding of ecosystem function
²⁰ and interactions (Bortolus (2008)). This is especially true for genera where identification down to species
²¹ can be useful as specific bioindicators; defining ecological and behavioral properties (e.g. different species
²² of Sagebrush- *Artemisia* L., Willows - *Salix* L., and Sedges - *Carex* L.) (Gage & Cooper (2013)). In these
²³ instances the lack of species level data can hinder our understanding of the breadth of habitat which some
²⁴ species occupy, and their interactions with other species. This can be further complicated by the fact that the
²⁵ identification of organisms to terminal taxon is also often mired by lack of diagnostic characters(e.g. flowers,
²⁶ fruits, roots or combinations thereof), an increasing lack of taxonomic experts (Hebert *et al.* (2003)) and
²⁷ the presence of cryptic species (Janzen *et al.* (2017), Oliver *et al.* (2009)). Taxonomic verification can
²⁸ also be limited by the fact that revisiting field sites to identify material using morphological or chemical
²⁹ approaches, can be resource intensive and often does not work. The current methods to ameliorate this
³⁰ situation include: ignoring these ecologically relevant levels of detail, revisiting plots as diagnostic material
³¹ becomes temporally available, seeking the assistance from taxonomic specialists, or the use of barcoding
³² using molecular techniques (CITE)

³³ Recently molecular barcoding (the identification of a sample from a single organism *e.g.* a piece of leaf),
³⁴ or metabarcoding (the identification of a sample containing a mix of organisms *e.g.* soil), have shown
³⁵ considerable promise in many taxa (Ruppert *et al.* (2019)). For plants the success is a little more mix,
³⁶ with the identification of certain clades using barcoding being quite successful (Kress (2017)), however for
³⁷ many other clades the results have been more elusive (Liu *et al.* (2014), Group *et al.* (2011), Coissac *et al.*
³⁸ (2012)), while metabarcoding has incurred additional challenges for the currently available barcodes (Li *et*
³⁹ *al.* (2015), Kress & Erickson (2007), Group *et al.* (2009), Coissac *et al.* (2012)). Particular challenges for
⁴⁰ the utilization of the high copy number barcodes (*e.g.* ITS2, *rbcL*, *matK*, *trnH-psbA*) include their rates of
⁴¹ divergence, gene tree conflict, and hybridization (Coissac *et al.* (2016), Fazekas *et al.* (2009)).

⁴² Currently the largest plant systematic endeavor ever undertaken,by the Royal Botanic Gardens Kew, the
⁴³ Plant and Fungal Tree of Life (PAFTOL) is approaching completion (Baker *et al.* (2021a)). This data
⁴⁴ set will contain hybridization capture (Hyb-Seq) data from at least one species in each genus of the plant
⁴⁵ kingdom,14,000 represented species, using the popular Angiosperms353 (A353) probes, which includes 353
⁴⁶ single-copy orthologous loci, (Baker *et al.* (2021a), Johnson *et al.* (2019)). These publicly available data
⁴⁷ serve to provide a taxonomically comprehensive backbone for plant metabarcoding. The A353 probes are
⁴⁸ currently being used in many other plant phylogenetic studies increasing the sampling depth of many clades

49 (Baker *et al.* (2021b)). Data from the 10kP project, which seeks to develop reference genomes from a
50 phylogenetically diverse suite of plants will contribute many more species upon it's intended completion,
51 slated for 2030. Similar projects such as the 'Darwin Tree of Life' which will sequence all described taxa in
52 Britain and Ireland, seek to sequence high amounts of genomes in geographic regions will contribute data
53 sets applicable to enormous spatial domains (Cheng *et al.* (2018), Life Project Consortium *et al.* (2022),
54 Lewin *et al.* (2022)). These data will promote the ability to apply metabarcoding to resolve a diverse array
55 of questions relevant to theoretical and applied ecology (Kress (2017), Hollingsworth *et al.* (2016)). However,
56 the application of metabarcoding still face challenges relating to the enormity of the genomic data sets and
57 the computational power required to process sequence data.

58 Herein we have resolved major components of the problems of identifying plant material without diagnostic
59 morphological character states using the A353 Hyb-Seq probes (Johnson *et al.* (2019)), and custom species
60 sequence databases derived via species distribution modelling, and temporal filtering.

61 To increase the quality of metabarcoding results in plants, we are proposing reducing the number of possible
62 candidate species by generating a user specific databases relevant to the region of study and the ecological
63 characteristics of interest (Bell *et al.* (2022)). To achieve this goal, we first create a regional list of candidate
64 species using digital collections gleaned from herbaria, survey work, and citizen science (e.g. iNaturalist),
65 from a region exceeding the study area. For these candidate species, a modelling approach, such as logistic
66 regression, may be used to identify taxa which warrant further exploration e.g. determine their possibility of
67 presence in metabarcoding samples. We then use species distribution models to create potential distribution
68 maps for the candidate species to limit the impact of spatial and taxonomic biases in the species list and
69 account for spatial variations in niche availability throughout the study area. Species distribution models
70 (SDM's) examine the ecological conditions associated with known occurrence of a species to identify where
71 in the study area might suitable habitats. This approach has the benefit of greatly reducing the size of a
72 sequence database, which allows for the usage of genomic size data on personal computers. This approach
73 can significantly reduce processing time, particularly as as most next-generation sequence data is deposited
74 as raw-sequence reads.

75 As species interactions vary both in space and time (@~THOMSON1994 GEO?, CaraDonna *et al.* (2021)).
76 Contrasts in the flowering periods of many plant species, can provide an additional filter for identifying
77 material in certain types of metagenomic samples (Janzen (1967), Newstrom *et al.* (1994)). In high elevation
78 temperate regions, pollination interactions vary temporally and are characterized by high turnover in active
79 periods of species (CaraDonna *et al.* (2017)), however the overall shorter extent of the active growing
80 season in these systems results in the presence of few to any natural breaks, which reduces the utility of

81 these to operate as filters in the post-processing of sequence matches. Nonetheless, we work develop a
82 general approach which seems applicable to many areas which utilize the temporal dimension for classifying
83 sequences in metagenomic samples (but see Davis *et al.* (2022)).

84 We tested this metagenomic and informatics approach to determine to examine the foraging behaviour of
85 queen bumblebee, and compare this approach with direct observations and the pollen record, which has shown
86 some incongruity in several floral visitation networks involving smaller bodied fauna (Barker & Arceo-
87 Gomez (2021), Zhao *et al.* (2019), Alarcón (2010)). The assessment of the plant species compositions from
88 pollen is a desirable approach, with several potential applications, despite numerous potential complications
89 (Poronon *et al.* (2017), Bell *et al.* (2017), Sickel *et al.* (2015), Bell *et al.* (2019), Suchan *et al.* (2019),
90 Johnson *et al.* (2021)). The two foraging phases of the queen bumblebee life cycle are essential to 1) increase
91 their weight before diapause, and 2) increase their ovary weights while establishing their recently found
92 nests. Both of these time periods represent potential demographic bottlenecks in bumble bee populations
93 (Sarro *et al.* (2022), ...). Bumblebees are one of the only groups of insects with unequivocal quantitative
94 evidence for numerous population declines, while simultaneously serving as the most effective pollinators
95 in many temperate montane ecosystems (Cameron & Sadd (2020), Goulson *et al.* (2008), Williams (1982),
96 Colla *et al.* (2012), Bergman *et al.* (1996), Bingham & Orthner (1998), Grixti *et al.* (2009)). These
97 areas often represent the most diverse areas in the temperate and often offer the sole potential refugia for
98 multiple dimensions of biodiversity under climate change, whilst simultaneously experiencing the greatest
99 proportional changes in mean annual temperature (Brito-Morales *et al.* (2018), Pepin *et al.* (2022)). An
100 immediate understanding of how to manage previously overlooked keystone insect species, such as bumble
101 bees, is essential if the refugial potential of the temperate mountains are to be incorporated into their current
102 diversity (Loarie *et al.* (2009), Dobrowski & Parks (2016)).

103 2 | METHODS

104 2.1 Study System & Field Work

105 Observations and bee sample collection was conducted at The Rocky Mountain Biological Laboratory
106 (RMBL; 38°57.5" N, 106°59.3" W (WGS 84), 2900 m.a.s.l.), Colorado, USA (APPENDIX 1 for site informa-
107 tion), characterized by high-montane/subalpine Parkland vegetation communities. Pollinator observations
108 of *Bombus* Latreille spp. (Apidae Latreille) were conducted from May 29th - July 23rd of 2015 in six study
109 sites. Observations of *Bombus* foraging took place for one hour at each field site in three 50m transects, each

transect was in a major vegetation type (dry, and wet meadows, and Aspen forest), where all flower abundances were estimated and placed into bins of abundance. Corbiculae loads were, non-lethally, collected once from all Queen individuals encountered.

2.2 | Floral Visitation

... does this need more info and warrant a section independent of the above?

2.3 | Pollen Morphological identification

2.3.1 | Pollen Reference Library

To develop a reference library of pollen grains which may be present in corbiculae loads, an image reference collection of fuchsin-jelly stained (Beattie (1971)) slides was assembled from slides previously prepared by the authors (n = 21), and other researchers (n = 38) (Brosi & Briggs (2013)). Using five years of observational data on *Bombus* Queen Bee foraging at these study sites (Ogilvie unpublished), as well as the Vascular Plant Checklist (Frase & Buck (2007)), an additional 62 voucher slides for species were prepared and imaged at 400x (Leica DMLB, Leica MC170 HD Camera, Leica Application Suite V. 4.13.0) from non accessioned herbarium collections to supplement the number of species and clades covered (Appendix 3).

We used Divisive Hierarchical Clustering techniques to determine which plant taxa were distinguishable via light microscopy, and to develop a dichotomous key to pollen morphotypes. Ten readily discernible categorical traits were collected from each specimen in the image collection. These traits were transformed using Gower distances, and clustered using Divisive Hierarchical clustering techniques (Maechler *et al.* (2022)). Using the cluster dendrogram, elbow plot, and heatmaps (Hennig (2020)), of these results morphological groups of pollen which could not be resolved via microscopy were delineated, and a dichotomous key was prepared (APPENDIX NO.). This key was then used to identify the pollen grains sampled from corbiculae loads to morphotypes in a consistent manner.

2.3.2 | Pollen Corbiculae Loads

To prepare the pollen slides from corbiculae, all corbiculae loads were broken apart and rolled using dissection needlepoints to increase heterogeneity of samples. *Cerca* 0.5mm² of pollen was placed onto a ~4mm² fuchsin jelly cube (Beattie (1971)) atop a graticulated microscope slide, with 20 transects and 20 rows (400 quadrants) (EMS, Hartfield, PA). The jelly was melted, with stirring, until pollen grains were homogeneously spread

137 across the microscope slide. Slides were sealed with Canada Balsam (Rublev Colours, Willits, CA) followed
138 by sealing with nail polish to prevent oxidation; all samples are noted in *APPENDIX 3*. To identify the pollen
139 present in corbiculae loads, light microscopy at 400x (Zeiss Axioscope A1) was used. In initial sampling in
140 three transects, each pollen grain was identified to morphotype and counted; an additional two transects
141 were scanned for morphotypes unique to that slide, if either transect contained an unique morphotype than
142 all grains in that transect were also identified and counted. Subsequent to the first round of sampling, non-
143 parametric species richness rarefaction curves (Oksanen *et al.* (2022)), and non-parametric species diversity
144 rarefaction curves were used to assess the completeness of sampling (Chao *et al.* (2014), Hsieh *et al.* (2020)).
145 Slides not approaching the asymptote of the rarefaction curve were then re-sampled, and analysed iteratively
146 for up to a total of seven transects *APPENDIX 2*.

147 2.4 | Molecular Barcoding

148 2.4.1 | Species reference list

149 **2.4.1.1 Spatial Analyses** We generate a short list of potential candidate species we downloaded from the
150 Botanical Information and Ecology Network ‘BIEN’ (Maitner (2022)) all records adjacent to the field sites
151 to develop an ecologically relevant list of vascular plant species, with expected biotic pollination, which may
152 be present at the study area. To reduce the list of species to include in the genomic sequence databases, we
153 then generated Species Distribution Models (SDMs) for these taxa to predict their distribution throughout
154 the study area.

155 In order to minimize the number of species for which SDM’s were to be generated, BIEN was queried at a
156 distance of up to 100km from our study area and all plant species records were downloaded. To account
157 for the stochasticity of botanical collecting and offset the number of records associated with the research
158 station, this data set was bootstrap re-sampled 250 times, with 90% of samples selected, to create a testing
159 data set. The median of the logistic regression assessing the probability of occurrence of a species record as
160 a function of distance from the study area was used as a threshold distance, under which, to include species
161 as candidates for distribution modelling.

162 **2.4.1.2 Distribution Modelling** We used all occurrence records from BIEN ($n = 23,919$) within a 50km
163 border of the Omernik level 3 ecoregion, which includes the study area (*No. 21 “Southern Rockies”*) to
164 construct the species distribution model (Omernik (1987)). These records were copied into two, initially
165 identical, sets, one for generating machine learning models (ML; Random Forest, and Boosted Regression

166 Tree's), and the other for Generalised Linear (GLM) and Generalized Additive Models (GAM) (Barbet-
167 Massin *et al.* (2012)). Ensembled predictions have been shown to outperform their constituent models, on
168 average, and to reduce the ecological signal to the analytical noise of individual runs (Araujo & New (2007)).
169 No single method of producing SDMs has been shown to universally outperform others when faced with
170 a large and diverse number of applications, in our case a great number of species with differing biologies
171 and ecologies (Elith* *et al.* (2006), Qiao *et al.* (2015)). In the spirit of these findings, multiple families of
172 models, which can be generated together as they have similar requirements regarding the number and ratios
173 of Presence to Absence records were ensembled together (Barbet-Massin *et al.* (2012)).

174 We then generated 4,029 absence points, locations where the focal taxon is anticipated missing, through a
175 random stratification of 19% of the land cover in the area and included them in (Land Management (2019)).
176 To achieve a larger absence data set, we generated 1,000 pseudo-absence records for each taxon by randomly
177 selecting coordinates located at least 10km away from an occurrence record. For ML models, these pseudo-
178 absences were reduced so that the ratio of presence to absence records were balanced (Barbet-Massin *et al.*
179 (2012)). To achieve this, we removed absence records inside of 10% of the mean sample value of any predictor
180 variable the presence records; the required number of absence records were then randomly sampled.

181 To predict the potential distribution of each species we used 26 environmental variables at 30m resolution,
182 six related to climate, five soil, four topographic, four related to cloud cover, with the remaining reflecting
183 assorted abiotic parameters (Wilson & Jetz (2016), Wang *et al.* (2016), Hengl *et al.* (2017), Robinson *et al.*
184 (2014)) (**APPENDIX 6**). **These publicly available data sets, were selected as they** For linear
185 regression models these predictors underwent both *vifstep* (theta = 10, max observations = 12,500) and
186 *vifcor* (theta = 0.7, max observations = 12,500) to detect highly correlated variables, and collinear features
187 were removed leaving 16 variables (Naimi *et al.* (2014)).

188 Modelling: Random Forest and Boosted Regression Trees, were sub sampled with 30% test and two replicates
189 each before weighted ensemble based on True Skill Statistics (tss) (Naimi & Araujo (2016)). Generalised
190 linear models (GLM) and Generalised additive models (GAM) with 30% sub sampling and three replicates
191 each were also ensembled using the tss (Naimi & Araujo (2016), @). TSS was chosen as the ensemble
192 criterion as it has been shown to work across a wide range of species occurrences prevalence (Allouche *et*
193 *al.* (2006)). The results of these models were extracted on a cell-by-cell basis to a polygon feature derived
194 from a minimum-spanning tree which encompasses the study sites, and species from either ensemble with
195 greater than 50% mean habitat suitability across all cells were considered present for further purposes (Prim
196 (1957)).

197 A total of 535 species were modelled using Generalized Linear Models and Generalized Additive Models and

198 534 species were modelled using Random Forest and Boosted Regression Trees. To evaluate the accuracy of
199 the species distribution models, additional presence records from GBIF ($n = 61,789$), and AIM ($n = 12,730$)
200 were used as test and training sets ($n = 74,519$) for logistic regression (Occdownload Gbif.Org (2021), Land
201 Management (2019)). Additional novel absence records were generated from the AIM data set to create a
202 data set where each species has balanced presence and absences. Eleven or more paired presence and absence
203 records were required for this testing, resulting in 334 species being included in the logistic regression (Mdn
204 = 110.0, $\bar{x} = 223.1$, max = 1568 record pairs used) with a 70% test split (Kuhn (2022)).

205 **2.4.2 | Temporal Analyses**

206 To estimate the duration of dates in which plant species were flowering weibull estimates of several pheno-
207 logical parameters all spatially modelled taxa were developed (Belitz *et al.* (2020), Pearse *et al.* (2017)).
208 Only BIEN records which occurred in the Omernik Level 4 Ecoregions within 15km of the study area ($n =$
209 5 Level 4 Ecoregions, or conditionally 6 ecoregions if enough records were not be found in the nearest 5),
210 and which were from herbarium records were included. To remove temporally irrelevant herbarium records,
211 i.e. material collected during times which flowering is impossible at the study area due to snow cover, we
212 used the SnowUS data set (Iler *et al.* (2021), Tran *et al.* (2019)) from 2000-2017 were analyzed for the first
213 three days of contiguous snow absence, and the first three days of contiguous snow cover in Fall. Herbarium
214 records after the 3rd quantile for melt, and the 1st quantile for snow cover of these metrics were removed.
215 Species with > 10 records had their weibull distributions generated for the date when 10% of individuals had
216 begun flowering, when 50% were flowering, and when 90% of individuals had flowered, we used the initiation
217 and cessation dates, respectively, as effective start and ends of flowering. These estimates were compared to
218 groundtruthed data from a long term experiment 1974-2012, and the field data from 2015, using kendall's
219 tau.

220 **2.5.2 | Barcode references library**

221 All lab work was carried out at The Daniel F. and Ada L. Rice Plant Conservation Science Center at the
222 Chicago Botanic Garden, Glencoe, Illinois, U.S.A.

223 **2.5.2.1 | Sampling Species for Barcoding** Using five years (2015-2020) of observational data on *Bombus*
224 Queen Bee foraging at these studies sites, we identified the plant taxa most frequently visited by Queens
225 across all years. We sequenced the 12 most commonly visited taxa twice using samples collected from one site
226 within the Gunnison Basin River Drainage and one individual collected from another more distal population.

227 In addition we included a congener - or a species from a closely related genus to serve as an outgroup for
228 all 12 taxa. In addition we sequenced another 15 taxa commonly visited by *Bombus* workers, based on the
229 abundances, and immediate access to plant tissue, in the aforementioned data set (*APPENDIX 4*). Plant
230 collections were identified typically using a combination, of dichotomous keys and primary literature as
231 required (Flora of North America Editorial Committee (1993+), Hitchcock & Cronquist (2018), Ackerfield
232 (2015), Lesica *et al.* (2012), Cronquist *et al.* (1977+), Allred & Ivey (2012), *Jepson flora project* (2020),
233 Mohlenbrock (2002)).

234 **2.5.2.2 | Plant Genomic DNA Extraction** Plant genomic DNA was isolated from ~ 1 cm² of leaf tissue
235 from silica-gel dried or herbarium material using a modified cetyltrimethylammonium (CTAB) protocol
236 (Doyle & Doyle (1987)) that included two chloroform washes. DNA was quantified using a Nanodrop 2000
237 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Qubit fluorometer (Thermo Fisher Scientific).

238 **2.5.2.3 | Pollen Genomic DNA Extraction** Pollen genomic DNA was extracted from corbiculae using
239 a CTAB based protocol modified from Lahlamgiah et al. and Guertler et al. (2014, 2014). A SDS extraction
240 buffer (350µL , 100mM Tris-HCl, 50 mM EDTA, 50 mM NaCl, 10% SDS v/v., pH 7.5) was added followed by
241 vortexing to allow dissolution of corbiculae. Pollen grains were then macerated with Kontes Pellet Pestles,
242 and the tip of these washed with 130 µL of the SDS extraction buffer, samples were then incubated for
243 1 hour at 30°C. This was followed by the addition of 10% CTAB solution (450ul, of 20 mM Tris-Cl pH.
244 8.0, 1.4 M NaCl, 10 mM EDTA pH 7.5, 10% CTAB, 5% PVP, ~85% Deionized water) and RNAse (10
245 uL of 10 mg/mL) and samples were incubated for 40 minutes at 37°C, on heat block (Multi-Blok, Thermo
246 Fisher Scientific, Waltham Massachusetts) set to 40°C. After 20 minutes incubation, Proteinase K (15 µL of
247 20mg/ml) and DTT (12.5 µL of 1M in water) were added, and the samples were further incubated at 60°C
248 for 1 hour. Samples were then incubated overnight at 40°C. 500 µL of Phenol-Chloroform-Isoamyl alcohol
249 (25:24:1) were added, vortexed, and centrifuged at 10,000 rpm for 10 minutes and the aqueous phase was
250 pipetted to a 1.5 ml centrifuge tube.

251 To precipitate the DNA, chilled Isopropyl alcohol & 3 mM Sodium acetate (5:1) equivalent to $\frac{2}{3}$ of the
252 volume of sample were added, with 1 hour of chilling at -20°C, followed by 10 minutes of centrifuging at
253 13,000 rpm. The supernatant was pipetted to a new 1.5 ml centrifuge tube, and 70% EtOH (400 µL) were
254 added before chilling at -20°C for 20 minutes followed by centrifugation at 13,000 rpm for 10 minutes. Both
255 tubes were then washed with 75% EtOH (400 µL), inverted, centrifuged at 13,000 rpm for 4 minutes, and
256 the solution discarded, then washed with 95% EtOH (400 µL) , inverted, centrifuged at 13,000 rpm for 4

257 minutes, and the solution discarded. Pellets were dried at room temperature overnight before resuspension
258 in nuclease free H₂O. Extractions were assessed using a Nanodrop 2000 (Thermo Fisher Scientific) and Qubit
259 fluorometer (Thermo Fisher Scientific). DNA extracts were then cleaned using 2:1 v./v. Sera-Mag beads
260 (Cytiva, Little Chalfont, UK) to solute ratio following the manufacturer's protocol, eluted in 0.5x TE, and
261 the eluent allowed to reduce by half volume in ambient conditions. DNA was quantified using a Qubit
262 fluorometer.

263 **2.5.2.4 | Fragmentation, Library Preparation & Target Enrichment** Library preparation was
264 performed using the NEBNext Ultra II FS-DNA Library Prep Kit for Illumina (New England BioLabs,
265 Ipswich, Massachusetts, USA) using slightly modified manufacturers recommendation. Fragmentation was
266 performed at ½ volume of reagents and ¼ enzyme mix for 40 minutes at 37°C, with an input of 500 ng cleaned
267 DNA. Adapter Ligation and PCR enrichment were performed with ½ volumes, while cleanup of products
268 was performed using SPRI beads (Beckman Coulter, Indianapolis, Indiana, USA) and recommended volumes
269 of 80% v./v. ethanol washes. The exception was the herbarium specimens which were not fragmented and
270 only end repaired, with similar library preparation of all samples. Products were analysed on 4% agarose
271 gels, and a Qubit fluorometer. Libraries were pooled and enriched with the Angiosperms 353 probe kit V.4
272 (Arbor Biosciences myBaits Target Sequence Capture Kit) by following the manufacturer's protocol and
273 Brewer et al. 2019. Sequencing was performed using an Illumina mi-Seq with 150-bp end reads, (NUSeq
274 Core, Chicago, Illinois).

275 **2.6 | Computational Processes and Analyses.**

276 **2.6.1 | Reference Library Data Processing** Sequences were processed using Trimmomatic, which
277 removed sequence adapters, clipped the first 3 bp, discarding reads less than 36 bp, and removing reads
278 if their average PHRED score dropped beneath 20 over a window of 5 bp (Bolger & Giorgi (2014), Tange
279 (2021)). Contigs generated were mapped to a reference with HybPiper with using target files created by
280 M353 (Johnson *et al.* (2016), McLay *et al.* (2021)).

281 **2.6.2 | Sequence Identification** A custom Kraken2 database was created by downloading representative
282 species indicated as being present in the study area by the spatial analyses from the Sequence Read Archive
283 (SRA) NCBI (Wood *et al.* (2019)). These sequences were processed in the same manner as our novel
284 sequences. The Kraken2 database was built using default parameters. Kraken2 was run on sequences using
285 default parameters (*APPENDIX 5*). Following Kraken2, Bracken was used to classify sequences to terminal

286 taxa (Lu *et al.* (2017)). Results from both Kraken2 and Bracken, results were reclassified manually to
287 identify terminal taxa. For example, when only a single species of a genus was known in the study area, but
288 our database used a representative of another taxon in the genus, this species was coded as the result.

289 **2.6.3 | Identification of Sequence Matching Loci** A local NCBI database was built using the same
290 processed novel and downloaded sequences as the previous database (Camacho *et al.* (2009)).

291 **2.7 | Integrated Observational, Molecular, and Palynological Corbiculae**

292 In order to precisely classify the contents of each corbiculae load the sequences classified by molecular methods
293 were compared with the fieldwork which at a very fine resolution, recorded the presence and absence of species
294 and their duration of flowering, and was interpreted ala the computer derived temporal and spatial data sets.
295 The quantitative counts of grains from microscopy, were combined with the semi-quantitative sequencing
296 results, to estimate the abundance of each identified species in each corbiculae load.

297 To reclassify the sequence reads, these data were combined with the flora observation data, and mapped
298 by genus. If more than one species in the genus was flowering at that time and site, than the reads were
299 split evenly between the taxa. For sequence data which did not match at the genus level, a user subjectively
300 scored them based on the species composition and phenological activity at each plot, the Queen interaction
301 data, and pollen assignments. To estimate the abundance of each of these species in the corbiculae loads,
302 these data were combined with the microscopy data. For each morphotype detected in pollen, and each
303 classified sequence read which was not detected via microscopy, they were given a value of 0.5% to indicate
304 there trace presences. When more than a single species belonged to a morphotype group in a single sample,
305 the quantitative values from the morphological work were multiplied by the relative sequence abundance of
306 each species in the load. All final compositions were standardized to a sum of 100%, by adding or subtracting
307 the differences (induced by classifying records as ‘trace’) to all species with abundances > 1%.

308 **3 | RESULTS**

309 **3.1 | Floral Observations**

310 The six sites were surveyed once weekly from May 27-July 27 for a total of 52 hours. A total of 723 queen-
311 pollen foraging interactions were observed (range per bee species by week range = 1-18, $\bar{x} = 3.46$, Mdn =
312 2), with a range of total observed interactions per bee species across this time period (min = 1, $\bar{x} = 59.08$,

313 Mdn = 19, max = 184). Plants varied widely in the number of interactions which they partook in with each
314 species of bee (range per plant species by week min = 1 - 20, $\bar{x} = 3.51$, Mdn = 2), with a range of total
315 observed interactions per plant species over this time period (min = 1, $\bar{x} = 20.26$, Mdn = 4, max = 141).
316 The number of plant species which bees were observed interacting with varied more narrowly (range = 1 -
317 18, $\bar{x} = 8$, Mdn = 6), interactions were observed with a total of 36 plant species.

318 A total of 66 corbiculae loads were collected from Bees, 64 of them from Queens.

319 [Figure 1 about here.]

320 3.1 | Spatial Analyses

321 [Table 1 about here.]

322 [Table 2 about here.]

323 The median (25.009 km) of the logistic regression assessing the probability of occurrence of a species record as
324 a function of distance from the study area was used as a threshold distance to include species for distribution
325 modelling. A 2-sample test for equality of proportions with continuity correction (X-squared = 13.254, df
326 = 1, p-value = 0.000136, 95% CI 0.04-1.00) was used to test whether more of the records located in the
327 broad ecological sites present at the field station, between the distance of the median (25.009 km) to the
328 third quantile (ca 43.830 km) of the regression distance, were true presences at the field station. Includinf
329 these records would have resulted in modelling an additional 222 species distributions of which 30 are true
330 presences, these taxa were not modelled.

331 Across the entire spatial domain of modelling all ensembled models ($n = 968$) had an accuracy of 0.84 (95%
332 CI 0.8356 - 0.8443), kappa = 0.68, p-value < 0.001, sensitivity = 0.80, specificity = 0.87, AUC = 0.92.

333 In the area of the minimum-spanning tree encompassing the field sites, of the 554 vascular plants with biotic
334 pollination syndromes, the 493 ML ensembles accurately predicted the presence of 362 (65.3%), incorrectly
335 predicted the presence of 64 (11.6%), incorrectly predicted 34 true presences (6.1%) as being absent, and
336 correctly predicted the true absence of 33 (6.0%). The balanced accuracy of the ensembled models is 0.627
337 (Sensitivity = 0.340, Specificity 0.914). Of the 554 vascular plants with biotic pollination syndromes, the
338 475 LM ensembles accurately predicted the presence of 286 (51.6%), incorrectly predicted the presence of
339 41 (14.3%), incorrectly predicted 93 true presences (16.8%) as being absent, and correctly predicted the
340 true absence of 55 (9.9%). The balanced accuracy of the ensembled models is 0.664 (Sensitivity = 0.573,

341 Specificity 0.754). Of the 554 vascular plants with biotic pollination syndromes in the flora 13 (2.3%) were
342 in the Orchid family and 41 (7.4%) are non-natives, both of which are restricted from the database, and can
343 only reduce the number of true predicted presences by roughly 10%.

344 At the six study plots, of the 117 plant species identified to the species level across the spatial extents of all
345 plots and duration of queen bee activity, the ML ensembles predicted the presence of 105 (89.7%) of them,
346 and LM ensembles 102 (87.2%). Of the missing species two (1.7%) are Orchids, six (5.1%) are non-native,
347 and one (0.85%) is of contested taxonomic standing, all of which (7.65%) are restricted from the initial query
348 database.

349 3.2 | Microscopic Pollen identification

350 Using the fuchsin jelly preparation and light microscopic analyses of grains and scoring of 10 character states
351 resulted in the establishment of 28 morphotypes which grains could be classified into. APPENDIX 7. From
352 the 37 samples that were counted and based on rarefaction we **had over % of expected morphotypes**
353 **found** (morphotype richness, $\bar{x} = 4.5$, Mdn = 4, min = 1, max = 9), **all samples had expected mor-**
354 **photype diversity reach the asymptote APPENDIX 8.** The number of counted pollen grains in each
355 sample range from (514 - 19924, $\bar{x} = 3319$, Mdn = 1891).

356 **SUMMARY REQUIRED - Number of species from number of families, and how many species**
357 **are identified to species versus number only to genera**

358 [Figure 2 about here.]

359 3.3 | Metabarcoding Pollen Identification

360 3.3.1 | Spatial Analyses to identify candidate taxa

361 The median (25.009 km) of the logistic regression assessing the probability of occurrence of a species record as
362 a function of distance from the study area was used as a threshold distance to include species for distribution
363 modelling. A 2-sample test for equality of proportions with continuity correction ($X^2 = 13.254$, df
364 = 1, p-value = 0.000136, 95% CI 0.04-1.00) was used to test whether more of the records located in the
365 broad ecological sites present at the field station, between the distance of the median (25.009 km) to the
366 third quantile (ca 43.830 km) of the regression distance, were true presences at the field station. Including
367 these records would have resulted in modelling an additional 222 species distributions of which 30 are true
368 presences, these taxa were not modelled.

369 Across the entire spatial domain of modelling all ensembled models ($n = 968$) had an accuracy of 0.84 (95%
370 CI 0.8356 - 0.8443), kappa = 0.68, p-value < 0.001, sensitivity = 0.80, specificity = 0.87, AUC = 0.92.

371 In the area of the minimum-spanning tree encompassing the field sites, of the 554 vascular plants with biotic
372 pollination syndromes, the 493 ML ensembles accurately predicted the presence of 362 (65.3%), incorrectly
373 predicted the presence of 64 (11.6%), incorrectly predicted 34 true presences (6.1%) as being absent, and
374 correctly predicted the true absence of 33 (6.0%). The balanced accuracy of the ensembled models is 0.627
375 (Sensitivity = 0.340, Specificity 0.914). Of the 554 vascular plants with biotic pollination syndromes, the
376 475 LM ensembles accurately predicted the presence of 286 (51.6%), incorrectly predicted the presence of
377 41 (14.3%), incorrectly predicted 93 true presences (16.8%) as being absent, and correctly predicted the
378 true absence of 55 (9.9%). The balanced accuracy of the ensembled models is 0.664 (Sensitivity = 0.573,
379 Specificity 0.754). Of the 554 vascular plants with biotic pollination syndromes in the flora 13 (2.3%) were
380 in the Orchid family and 41 (7.4%) are non-natives, both of which are restricted from the database, and can
381 only reduce the number of true predicted presences by roughly 10%.

382 At the six study plots, of the 117 plant species identified to the species level across the spatial extents of all
383 plots and duration of queen bee activity, the ML ensembles predicted the presence of 105 (89.7%) of them,
384 and LM ensembles 102 (87.2%). Of the missing species two (1.7%) are Orchids, six (5.1%) are non-native,
385 and one (0.85%) is of contested taxonomic standing, all of which (7.65%) are restricted from the initial query
386 database.

387 3.3.2 | Temporal Analysis

388 The first date of modeled snow melt in the Gothic area ($n = 17$, $\bar{x} = 137.9$, Mdn = 135, 3rd quartile =
389 151), and the first date of a consistent winter snow base ($n = 17$, $\bar{x} = 299.9$, Mdn = 300, 1st quartile =
390 291) from 2000-2017, were used as delimiters for the inclusions of herbarium records in modelling. Of the
391 439 species predicted likely present in the area via logistic regression, 332 species (64.4%) with more than 10
392 records in the focal level 4 ecoregions ($\bar{x} = 35.016$, Mdn = 35, max = 96) had weibull estimates calculated,
393 an additional 56 species (11.2%) with enough contributing records from the “Sedimentary Mid-Elevation
394 Forests”, a large ecoregion generally just beneath the elevation bands occupied by the five ecoregions around
395 the study area had weibull estimates also calculated ($\bar{x} = 13.868$, Mdn = 13, max = 24).

396 Only 58 of these 388 species ($n = 34.6$, Mdn = 31) were able to be compared to plot based observational data
397 from the long term (1974–2012) data set (CaraDonna *et al.* (2014)). Of these species relatively high accord
398 was observed between the long-term ground truthed data set, and the modelled species. There was very

399 strong evidence that the weibull estimates were positively associated with the observed onset ($p < 0.0001$,
400 $\tau = 0.61$), peak ($p < 0.0001$, $\tau = 0.65$), and cessation of flowering ($p < 0.0001$, $\tau = 0.49$). There was
401 moderate evidence that the weibull estimates had a weak positive association with the observed duration of
402 flowering ($p = 0.58$, $\tau = 0.17$).

403 Of the previous 58 species compared, 47 of these could be compared to plot based data from the six sites
404 observed in 2015. Due to methodological differences, the peak flowering was not compared, and due to the
405 low performance of attempts to model ‘duration’ in the previous step it was also not compared. There was
406 very strong evidence that the weibull estimates were positively associated with the observed onset ($p <$
407 0.0001 , $\tau = 0.58$), and cessation of flowering ($p < 0.0001$, $\tau = 0.40$).

408 [Figure 3 about here.]

409 [Figure 4 about here.]

410 3.3.1 | Molecular analysis of corbiculae loads

411 The 54 corbiculae loads had DNA extracted and underwent various steps towards hyb-seq, in the end a total
412 of 44 corbiculae samples were sequenced, 7,752,353 reads were recovered from sequencing. The number of
413 reads per sequence varied widely (range = 76 - 508,795, $\bar{x} = 176,189.8$, Mdn = 138,395). Of the possible 353
414 loci, the number which were recovered from each sample, and informative to BLAST were range = 24 - 353,
415 $\bar{x} = 305.5$, Mdn = 331. The number of reads per loci from across all samples had a range of 178 - 506,653,
416 $\bar{x} = 20,688$, Mdn = 12,616. **APPENDIX X Reads Per Loci.**

417 After trimming 7,865,680 sequences remained. 10,682,538 reads were matched using Kraken, of the reads
418 classified by Kraken 10,160,768 reads were matched using Bracken, of the reads classified by Kraken 7,549,608
419 reads were matched using BLAST. Based upon subjective review of the three classifiers **APPENDIX X**
420 **MOLECULAR NETWORKS - 3 DIFFERENT ONES**, BLAST was chosen as the classification
421 method which yielded the most probable results by the field ecologist, and it’s values were used for all
422 subsequent analyses.

423 [Table 3 about here.]

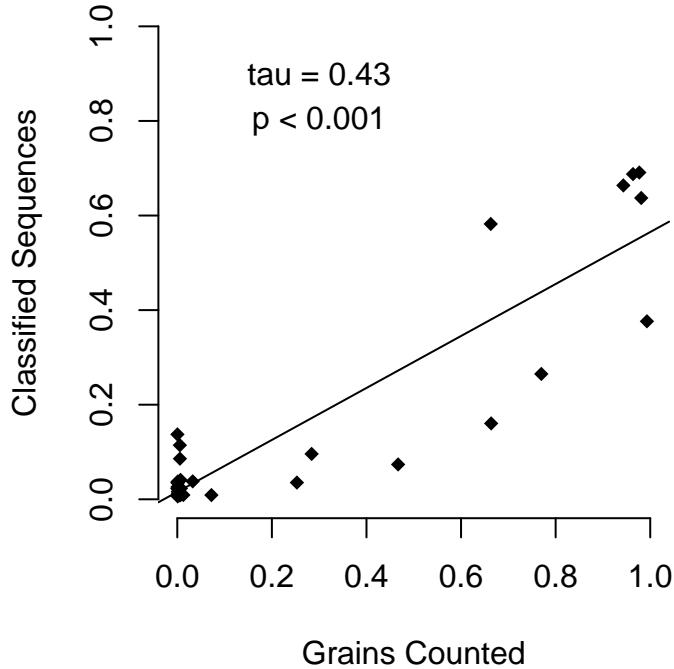
424 The initial classification of sequences which were made by BLAST were reviewed programmatically, using
425 predicted presence of the species (from spatial modelling), modelled flowering time (from temporal mod-
426 elling), and taxonomy (from existing sources). A sequential process was utilized which reassigned sequences

427 based on binary combinations of the factors above (Appendix XX). Given the relative sparsity of the number,
428 and relatedness, of species represented in the sequence database this was performed to: 1) Identify locally
429 present species represented by surrogates in the DB 2) Reduce false classifications of focal species 3) Identify
430 high confidence sequence matches. Of the top ten taxa which were identified by BLAST for the 680 distinct
431 records, 55.4% of the reads were classified to a species representing 48.3% of all classified reads, 41.9% of the
432 reads were classified to genus representing 48.3% of all classified reads, and 0% of the records were classified
433 to family.

434 Of the 0 classifications which were assigned to genera without any species predicted by spatial analyses, were
435 investigated by hand after post-processing steps. These were all assigned via post-processing conditions (: ,
436 APPENDIX XX). These were manually assigned to a variety of ranks, occasionally to genus - 0, and species
437 - 0, by consultation of the alpha-taxonomic literature (Sadeghian *et al.* (2015), Sennikov & Kurtto (2017),
438 Rabeler & Wagner (2016), Pusalkar & Singh (2015), Moore & Bohs (2003), Weber (1998)).

439 To determine at which level species in pollen loads could be detected the results of light microscopy were
440 compared to the molecular results. The pollen samples contained three morphotypes which could readily
441 be identified via microscopy. Two of these mapped to the clades (Boraginaceae & Heliantheae Alliance),
442 and one to a Asteraceae less Heliantheae. Boraginaceae grains were detected in 92.3% of samples where the
443 proportion of target grains were between 0.01-1 ($n = 13$ Mdn = 0.663). Asteraceae type 1, non-helianthoids,
444 were detected in 50% of samples where the proportion of target grains were between 0.001-0.01 ($n = 4$ Mdn
445 = 0.001) Asteraceae type 2, Helianthoids, were detected in 33.3% of samples where the proportion of target
446 grains were between 0.001-0.01 ($n = 6$ Mdn = 0.005); however, Asteraceae were detected in 80% of samples
447 where the proportion of target grains were between 0.001-0.01 ($n = 10$ Mdn = 0.003). Both morphotypes
448 of Asteraceae pollen were detected in 100% of samples where the proportion of target grains were between
449 0.01-1 ($n = 2$ Mdn = 0.338).

Correlation of Proportion Counted Grains and Sequence Reads



450

To detect whether the sequencing reads were semi-quantitative the subset of all pollen morphotypes distinguishable by microscopy were compared to the sequence reads. In all instances sequence reads were pooled to the highest taxonomic rank associated with the morphotype, e.g. if both species of *Mertensia* Huth, or one species and read only classified to genus were present in a sample, the reads were summed. The total percentage of the ten most abundant grains per sample were then relativized to constitute the entire sample.

The relationship between the number of pollen grains in a sample and the number of sequence reads is roughly linear, where grains which are present in trace amounts are overestimated by sequence counts, while grains present in high amounts are underestimated. This is likely due to the proportion of high false positives which occur in the classification process with next-generation sequencing (Bell *et al.* (2021)). There was evidence of a strong correlation between the proportion of grains per morphotype and the number of sequences per group (0.43 , $p < 0.0001$, $n = 32$).

To ascertain the extent to which records of multiple species in a family, which were suspected to be sampling artefacts occurred in molecular samples an index of similarity, ala jaccard, the affinity index was used to assess co-occurrence (Mainali *et al.* (2022), Mainali & Slud (2022)). Numerous taxa from the family Ranunculaceae Jussieu (*Caltha* L. sp., *Thalictrum* L. spp., *Trollius* L. sp., *Aquilegia* L. spp.), had α scores which indicated that they are only present when a more common confamilial taxa *Delphinium barbeyi* (Huth)

468 Huth *nuttallianum* Pritz. were recorded. A similar relationship was observed in the Hydrophyllaceae R.Br.
469 with samples placed in *Nemophila* Nutt., which only occurred when the more abundant *Hydrophyllum* L.
470 species were present. The size of flower of *Nemophila breviflora* A. Gray make it unlikely to be visited by
471 Bumble Bees, and it is a false positive. The floral morphology of *Thalictrum* spp. also makes it unlikely to
472 be visited (BELEIVE JANE SAID THIS- IF NOT STRIKE OUT), and while evidence of visits to *Caltha*
473 and *Trollius* are lacking, due to the association between the reads these results appear unlikely.

474 3.6 | Integrated Observational, Molecular, and Palynological Network

475 While the spatial results were used to declare the taxonomic composition of the sequence database, temporal
476 results were used in consideration with plant phylogeny to retroactively, reassign the assignment of sequences
477 to taxa. Essentially, if a sequence was identified to a taxon which was not known from the field site

478 For example a many sequences which mapped to the Asteraceae family, but which was flagged by temporal
479 filters and is present in both *B. nevadensis* Cresson and *B. rufocinctus* Cresson pollen is most likely *Frasera*
480 Walter, which failed extractions for the reference library failed (APPENDIX XX). A similar likely mismatch
481 could be between what was fide molecular evidence as *Agastache pallidiflora* (A. Heller) Rydb. but where
482 feeding was infrequently observed on *Pedicularis* L., likely due to this entire order being represented by only
483 a single molecular reference species.

484 Situations where SDM's led to incorrect results at the species level are evident with classification to *Scabrethia*
485 *Scabra* (Hooker) W.A. Weber, this match almost certainly representing *Wyethia arizonica* A. Gray (Weber
486 (1998)), a taxon known to be visited by Queen bee's via our floral observations. An expected inaccuracy of
487 the classification scheme is in genus level placements, e.g. were *Epilobium* L. (Onagraceae Juss.) spp. were
488 classified. However, given the small size of their flowers in the study area, these results more likely indicate
489 that a species of *Chamaenerion* Seg. (a segregate genus) such as *C. angustifolium* (L.) Scop. or *latifolium*
490 (L.) Sweet is occasionally utilized, as it supported by limited palynology data. An issue with reclassification
491 within the family level in combination with time included reclassifying *Parnassia palustris* L. to *Paxistima*
492 *myrsinoides* (Pursh) Rafinesque. However, based on flower size it is more likely that the visited taxon was *P.*
493 *palustris*.

494 Regarding limitations of morphological data we suspect that there were two morphotypes of pollen identified
495 as Ericaceae Juss. were actually Onagraceae (Samples 19 & 44), based on molecular results.

496 It is not unlikely that much of the difference in the results between the observational and molecular work
497 are attributable to the challenges in detecting rare events in these smaller sizes. For example, no more than

498 10 bee corbiculae loads per species were sequenced with the Mdn = 5.5 . . . , and the median of interactions
499 with the top 5 plant sizes constituted 0.8283385 of the top interactions. Accordingly, combining the results
500 of floral observations, and palynology, molecular sequencing - both pre and post processing, we subjectively
501 developed re-classifications of the contents of pollen grains . . .

502 4 | DISCUSSION

503 We have demonstrated how the Angiosperms533 hyb-seq probes may be used for plant barcoding in a
504 metagenomic context (Johnson *et al.* (2019), Hollingsworth *et al.* (2016)). This was exemplified in an
505 ecologically relevant scenario, where the results have immediate implications for natural history guided
506 fundamental science and land management. The test pollen loads contained a number of closely related
507 taxa, some in notoriously morphologically difficult clades with rapid rates of diversification (e.g. *Mertensia*,
508 *Lupinus* L.), at naturally occurring proportions (Nevado *et al.* (2016), Nazaire & Hufford (2014)). We
509 incorporated spatial and temporal approaches for creating custom sequence databases an approach which
510 is readily applicable to any lab group with the capacity to perform next-generation sequencing across the
511 entirety of multiple continents, and which we expect to be highly beneficial in many study areas. By
512 combining insights from these novel approaches with an extensive observational field based study we show
513 how these methods may be applied to test a variety of hypotheses related to ecological interactions.

514 The SDM's which we generated, with relatively few occurrence records and few modelling iterations, per-
515 formed beyond expectations, likely due to the utility of the predictor variables and strong alignment of
516 vegetation by orographic precipitation in the study area. However, we had difficulties in evaluating our
517 predictions in an operational context. We utilized the database query approach, to only model species with
518 a high probability of not being dispersal limited to the focal area, and focused on a relevant subset of many
519 of these species ranges to reduce the contributions of range wide adaptions on habitat (Sork (2018), Joshi
520 *et al.* (2001)). While the models worked well compared to both test, and validation with external point
521 data, moving from points to polygon features was more difficult. We were able to compare our results to
522 1) a Flora, 2) lists of plants used by Bumble Bees at plots; the former inappropriate in that it contained
523 a great number of species which we sought to use modelling to reduce *e.g.* all strictly alpine species, and
524 the latter inappropriate in that it contained only species relevant to *Bombus* but had no official 'absence'
525 data. Further given the, size of the minimum spanning tree which we extracted points to, a formal floristic
526 inventory would still be a time intensive process. Accordingly, we expect the real results of our data lay
527 somewhere in between these two evaluations; with an excess of species predicted present (Dubuis *et al.*

528 (2011), Calabrese *et al.* (2014), Pinto-Ledezma & Cavender-Bares (2021)), but few enough that they lend
529 themselves to metabarcoding. We observe that our models seemed very capable of effectively identifying
530 alpine species and removing them in binomial contexts. Difficulties in temporal models related to variability
531 in drivers of flowering phenology. AND SMALL SAMPLE SIZE

532 These results show that the overall results between **Bumble Bee ecology** observational and barcoding are
533 largely congruent. But that ... We analyzed pollen loads from all of the most common bumble bee species
534 in the area (Pyke (1982)) Future analyses of the long term data set...

535 Results from palynological analyses show that several species of bee show near perfect fidelity to the genus
536 *Mertensia* on a per visit basis... General results show high congruence between foraging and molecular
537 results, indicating that concerns regarding mismatch between observational networks need not persist with
538 *Bombus* studies...

539 Some foraging preferences of *Bombus*, both at this field site and across a great many localities globally
540 emerge from this work, which reiterates the needs for land managers to maintain relatively high amounts
541 of members of the Fabaceae, Boraginaceae, and Ranunculaceae, in Western North American montane land-
542 scapes (Goulson *et al.* (2005), Goulson (2010), Liang *et al.* (2021), Bontsutsnaja *et al.* (2021)). Numerous
543 historic, and some ongoing, land management practices reduce the ability of many landscapes to support
544 stable populations of *Bombus*. Historic livestock grazing was often associated with the targeted removal
545 of many species of plants which are known to have compounds toxic to cattle. In particular, the removal
546 of locoweeds (Fabaceae: *Astragalus* L. & *Oxytropis* DC.) and larkspurs (Ranunculaceae: *Delphinium*) were
547 common across public lands administered by the United States Forest Service (Ralphs & Ueckert (1988),
548 Aldous (1919), Ralphs *et al.* (2003)). Further actions, generally initiated by early settlers, involved the
549 channelization and incising of streams, culling of beavers, and leaving cattle concentrated on higher order
550 stream banks for significant periods of time, all processes which lower the water tables and reduced the ex-
551 tent of stream-associated [riverine] wetlands and the mesic meadows fringes which provide habitat for many
552 species of tall *Mertensia* (Boraginaceae, e.g. *M. ciliata* Torr. G. Don.) widely distributed across Western
553 North America, and to an extent *Delphinium barbeyi* and many species of native *Trifolium* L. (Dahl (1990),
554 Naiman *et al.* (1988), Belsky *et al.* (1999), Cooke & Reeves (1976)). Fire suppression further resulted in the
555 succession of many Aspen (*Populus tremuloides* Michx.) groves to Conifer stands, decreasing the mosaic of
556 age structured habitats in many landscapes, adversely effects habitat for tall *Mertensia* species and several
557 species of *Delphinium* (Brewen *et al.* (2021), Keane (2002)). Finally the effects of Nitrogen deposition, es-
558 pecially given the West's rapidly growing population still pose adverse effects on the abundance of a variety
559 of species of Fabaceae at Urban-Rural interfaces (see Stevens *et al.* (2018), Fenn *et al.* (2003)). Current

560 solutions to these issues, involve targeted burns, reintroduction of beavers and beaver habitat analogs, and
561 the possibility of re-seeding a variety of ‘locoweeds’ and ‘larkspurs’ in areas now seldom used, or only used
562 for early, grazing. The highly enthusiastic response of land managers, and homeowners, to plant *Asclepias*
563 L., using genetically appropriate materials, to improve Monarch Butterfly (*Danaus plexippus* L.) habitat
564 provides an effective framework for the latter (Oberhauser *et al.* (2015), Basey *et al.* (2015)).

565 We have concerns regarding the number of persons training to become and practice botany, and grave
566 concerns regarding the funding mechanisms for floristic and field based botanical research and for centralized
567 authorities to produce consensus opinions on alpha taxonomy (Prather *et al.* (2004b), Kramer & Havens
568 (2015), Prather *et al.* (2004a), Crisci *et al.* (2020), Manzano (2021), Stroud *et al.* (2022)). To reduce
569 the effects of a low population density of botanists on the maintenance of and production of Flora’s and
570 to foster meta-genomics across landscapes without field stations we utilized Species Distribution Modelling
571 to generate predictive species lists. In this proof of concept example we performed several iterations of
572 modelling runs, and several approaches (i.e. the ‘linear models’, and the ‘machine learning’), which took
573 notable amounts of compute power. We suspect the possible deleterious nature of this endeavor may be
574 reduced by: 1) more field surveying by crews will reduce the need to generate as many species 2) fewer runs
575 of models, 3) only running machine learning models which do not require an explicitly process to reduce
576 spatial autocorrelation. However, given the time required to perform all aspects of a study, even our amount
577 of computation was negligible. Further, we are very optimistic about the possibility for persons to perform
578 these tasks, as mentioned we utilized roughly only one quarter of the records which were digitally available
579 for presence, and we suspect others will have enough records to perform this process nearly anywhere else in
580 the temperate. In certain scenarios modelling of predicted species via more formally tailored S(tacked)-SDM
581 or J(oint)-SDM approaches may be beneficial (Wilkinson *et al.* (2021), Pinto-Ledezma & Cavender-Bares
582 (2021), Schmitt *et al.* (2017)).

583 Tandem to the lack of continued expertise required to generate and maintain species lists, is the expertise
584 required to continue tracking when major phenological events occur in many plant species at relatively fine
585 scales or under novel climates. Knowledge of these events is currently limited to general time periods of only
586 a handful of phenological events and groups of organisms (e.g. flowering initiation, or trees) (Prather *et al.*
587 (2004a), Li *et al.* (2016)). While many programs and initiatives exist to collect phenological information on
588 subsets of easily identifiable charismatic species to detect major trends in phenology, these capture only a
589 subset of the extent diversity (Betancourt *et al.* (2005), Havens *et al.* (2007)). In many instances it appears
590 that while landscapes respond similarly to environmental variables which predict phenological responses,
591 that individual species vary widely in their responses to similar environmental cues, or respond to different

592 cues (Augspurger & Zaya (2020), Xie *et al.* (2015), Xie *et al.* (2018), CaraDonna *et al.* (2014)). As can
593 be seen here, predictions of when a single, major phenological event occurs is already data limited. A more
594 promising approach for the tropics may lay in utilizing circular statistics (Park *et al.* (2022)).

595 The nearly complete Plant and Fungal Tree of Life (PAFTOL) will provide a comprehensive phylogenetic
596 backbone of the entire plant kingdom, and the inclusion of A353 probes with lineage specific probe sets is
597 common in producing massive genetic datasets (Baker *et al.* (2021b)). We predict that the A353 probes
598 which it is utilizing to work nearly immediately for DNA barcoding of whole plant material, and that more
599 elaborate validation studies in controlled metabarcoding settings, utilizing existing experimental designs,
600 will have favorable results (Bell *et al.* (2017), Bell *et al.* (2019), Bell *et al.* (2021), Lamb *et al.* (2019)). In
601 particular the harvesting of loci with more variation in certain lineages, and or with more variable flanking
602 regions, will prove promising for identifying closely related plant material (CITE). We suspect that conserved
603 reaches of genes resulted in the high amounts of reads in somewhat obscure species. Given that the A353 loci
604 are nuclear, single copy, and a variety are present the possibility of identifying target loci for quantitative
605 purposes is high, without continual PCR enrichment is possible; this would align with relatively high efficacy
606 of WGS (Lang *et al.* (2019), Peel *et al.* (2019), Bell *et al.* (2021)). Recent evidence indicates that the
607 potential for identifying nearly cryptic taxa and even infra-specific inference, of either whole plant material,
608 and perhaps in metagenomic context are possible (Ottenlips *et al.* (2021), Wenzell *et al.* (2021), Loke et
609 al. in prep, Slimp *et al.* (2021), Beck *et al.* (2021)). We further believe that in synthetic phylogenetic
610 trees - with incorporation of NGS backbones - will allow in automatic reassignment of reads as a function of
611 phylogenetic distance with measures of uncertainty (Hinchliff *et al.* (2015), Smith & Brown (2018), Baker
612 *et al.* (2021a)).

613 5 | CONCLUSION

614 We believe that the combination of spatial and temporal models, united and guided by localized natural
615 history knowledge, provides the essential components of a bayesian framework for approaching the coarse
616 elucidation of ecological interactions using DNA Barcoding. Herein we crudely utilized this thinking via
617 binary outcomes, should a species predicted be predicted present or not? Is it unequivocally flowering
618 or not? Myriad data show biological systems and ecological interactions have more variance than can be
619 reasonably discretely parsed. We expect that within a bayesian framework studies of pollinator behavior
620 may be enacted via this approach at a landscape level, e.g. the scale of an entire drainage basin such as the
621 Gunnison which is quickly becoming one of the worlds few model ecosystems. We hope that the promise of

622 A353 probes as tools for metabarcoding play a role in these endeavors.

623 **AUTHOR CONTRIBUTIONS:** R.C.B conducted botanical collections, conducted all molecular lab
624 work, lead all analyses, and writing. J.E.O conceived, designed, and conducted all ecological fieldwork,
625 assisted with analyses, and writing. E.J.W. prepared, imaged, and collected trait data on pollen reference
626 slides, and assisted with analysis of trait data and writing a dichotomous key. S.T. assisted with spatial
627 analyses and writing. P.J.C assisted with ecological analyses and writing. J.B.F. conceived, and designed all
628 lab work, analyses, and integration of approaches, assisted with writing, and secured funding for molecular
629 work.

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643 AK, ALTA, ASU, BABY, BC, BM, BMO, BOON, BRIT, CANB, CAS, CHSC, CM, CMN, CNS, COLO,
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645 LOB, LSU, MA, MACF, MEL, MICH, MIL, MIN, MNHN, MO, MO, MT, MW, NCSC, NSW, NY, NYBG,
646 O, OBI, PI, RBG, RSA, SD, SDSU, SFV, TENN, TRT, UA, UAC, UAM, UAZ, UBC, UBC, UCR, UCS,
647 UCSB, UMO, UNM, UPS, US, USCH, USF, USU, UTEP, UWBM, V, VT, W, WSCO, WU, XAL, YPM,
648 Z.

649 **CONFLICT OF INTERESTS** The authors declare no conflicts of interest.

650 **PEER REVIEW** The peer review history for this document is available at ...

651 **DATA AVAILABILITY STATEMENT** The queries required to download all data used in this project
652 are located in... All novel sequencing data are located at NCBI...

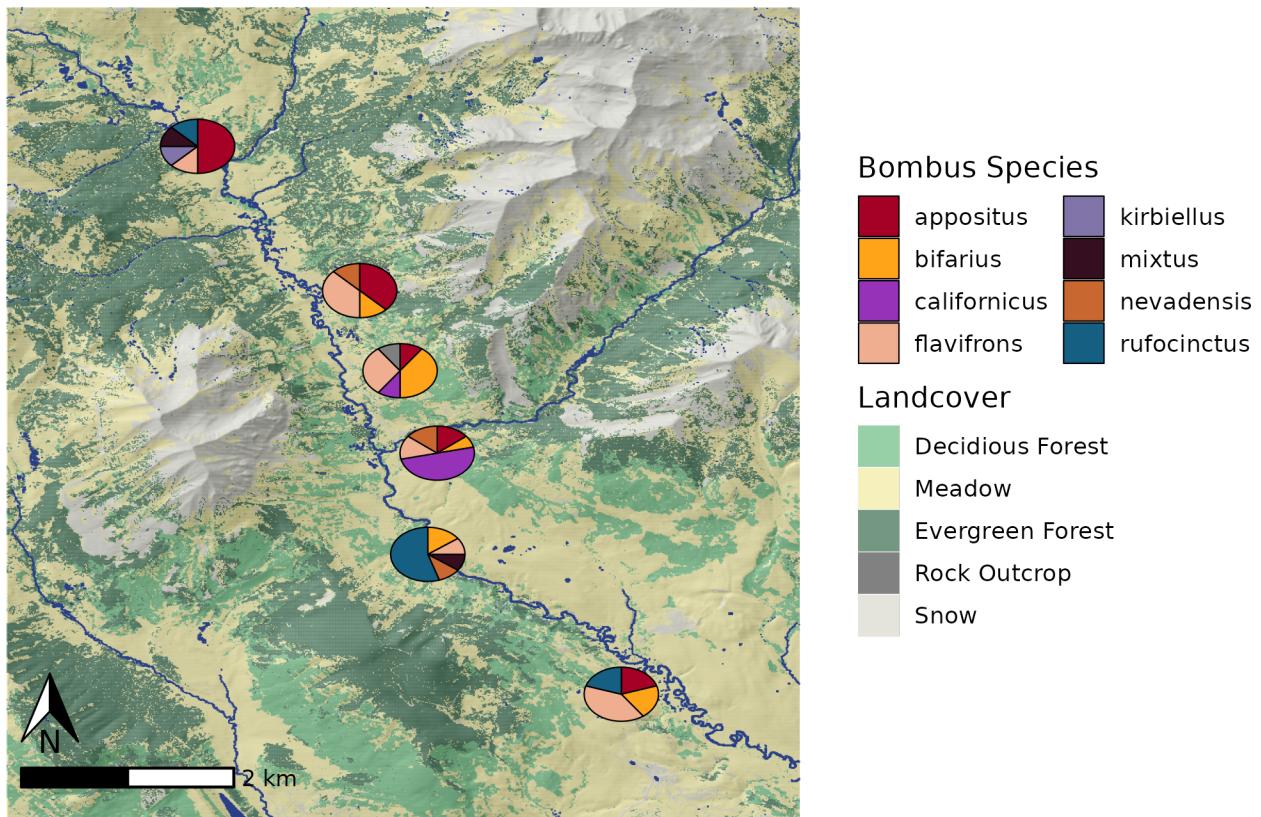
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658 **References**

659 **Supporting**

Origins of Corbiculae Loads



Upper East River Valley, Colorado

Layer	LM	Description	Source
1.	N	Mean annual cloudiness - MODIS	Wilson et al. 2016
2.	Y	Cloudiness seasonality 1 - MODIS	Wilson et al. 2016
3.	N	Cloudiness seasonality 2 - MODIS	Wilson et al. 2016
4.	Y	Cloudiness seasonality 3 - MODIS	Wilson et al. 2016
5.	N	Beginning of the frost-free period	Wang et al.
6.	N	Climatic moisture deficit	Wang et al.
7.	N	Degree-days above 5C	Wang et al.
8.	N	Mean annual precipitation	Wang et al.
9.	Y	Mean annual precipitation as snow	Wang et al.
10.	Y	Temperature seasonality	Wang et al.
11.	Y	2015 Percent Grass/Herbaceous cover - MODIS	(MOD44B)
12.	Y	2015 Percent Tree cover from Landsat 7/8	(GLCF)
13.	Y	Soil probability of bedrock (R Horizon)	SoilGrids
14.	N	Soil organic carbon (Tonnes / ha)	SoilGrids
15.	N	Surface soil pH in H ₂ O	SoilGrids
16.	Y	Surface soil percent sand	SoilGrids
17.	Y	Soil USDA class	SoilGrids
18.	N	Topographic elevation	EarthEnv DEM
19.	Y	Topographic elevation, moving window.	EarthEnv DEM
20.	Y	Topographic percent slope	EarthEnv DEM
21.	Y	Topographic wetness index	EarthEnv DEM
22.	Y	Topographic aspect	EarthEnv DEM
23.	Y	Annual potential solar radiation computed	r.sun
24.	N	Estimated actual (w/-cloud) solar radiation	r.sun / Wilson et al. 2016
25.	Y	Log-transformed distance to surface water	Global Surface Water Explorer
26.	Y	Percent surface water	Global Surface Water Explorer

Table 1: samples used in creating the Reference Library

Taxon	Family	Accession	Pres.	Locality	Date Col.	GenBank	Dist. (km)
<i>Cirsium parryi</i> (A. Gray) Petr.	Asteraceae	CHIC tba	S	Colorado, Gunnison	20.IX.2020	tba	3.6
<i>Cirsium parryi</i> (A. Gray) Petr.	Asteraceae	CHIC tba	S	Colorado, Gunnison	20.IX.2020	tba	3.6
<i>Ericameria parryi</i> (A. Gray) G.L. Nesom & Baird	Asteraceae	CHIC tba	S	Colorado, Gunnison	20.IX.2020	tba	3.6
<i>Erigeron speciosus</i> (Lindley) De Candolle	Asteraceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Erigeron subtrinervis</i> Rydb. Ex Porter & Britton	Asteraceae	CHIC tba	S	Colorado, Gunnison	20.VII.2020	tba	3.6
<i>Helianthella quinquenervis</i> (Hook.) A. Gray	Asteraceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Helianomeris multiflora</i> Nutt.	Asteraceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Heterotheca villosa</i> (Pursh) Shinners	Asteraceae	CHIC tba	S	Colorado, Gunnison	20.IX.2020	tba	3.6
<i>Senecio sera</i> Hook.	Asteraceae	CHIC tba	P	Idaho, Idaho	26.VII.2020	tba	105.0
<i>Symplytrichum foliacum</i> (Lindl. Ex D.C.) G.L. Nesom	Asteraceae	CHIC tba	S	Illinois, McHenry	28.VII.2020	tba	1624.6
<i>Taraxacum officinale</i> F.H. Wigg.	Asteraceae	CHIC tba	S	Illinois, McHenry	28.VII.2020	tba	1624.6
<i>Mertenia ciliata</i> (James ex Torr.) G. Don	Boraginaceae	ID 1754185	S	Idaho, Valley	18.VI.2018	tba	979.3
<i>Mertenia ciliata</i> (James ex Torr.) G. Don	Boraginaceae	ID 169837	P	Idaho, Adams	10.VII.2014	tba	991.5
<i>Mertensia fusiformis</i> Greene	Boraginaceae	RMH 720522	P	Colorado, Gunnison	7.VI.1997	tba	44.8
<i>Campanula rotundifolia</i> L.	Campanulaceae	RMH 720600	P	Colorado, Gunnison	9.VII.1997	tba	38.9
<i>Lathyrus lanszwertii</i> Kellogg var. leucanthus (Ryd.) Dorn	Fabaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Lathyrus lanszwertii</i> Kellogg var. leucanthus (Ryd.) Dorn	Fabaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Lupinus argenteus</i> Pursh	Fabaceae	CHIC tba	P	Nevada, Pershing	29.V.2018	tba	971.2
<i>Lupinus argenteus</i> Pursh	Fabaceae	ISU 10387	P	Colorado, Gunnison	29.VI.2010	tba	0.2
<i>Lupinus bakeri</i> Greene	Fabaceae	ISU 10142	P	Colorado, Gunnison	15.VIII.2010	tba	2.6
<i>Vicia americana</i> Muhl. ex Willd.	Fabaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Vicia americana</i> Muhl. ex Willd. var. minor Hook.	Fabaceae	CHIC tba	S	Montana, Carbon	4.VII.2019	tba	10020.8
<i>Frasera speciosa</i> Douglas ex Griseb	Gentianaceae	RMH 721930	P	Colorado, Gunnison	20.VI.1997	tba	66.2
<i>Frasera speciosa</i> Douglas ex Griseb	Gentianaceae	RMH 719305	P	Colorado, Gunnison	7.VII.1997	tba	19.8
<i>Hydrophyllum capitatum</i> Douglas ex. Benth	Hydrophyllaceae	RMH tba	P	Colorado, Mesa	30.VI.2011	tba	64.6
<i>Hydrophyllum capitatum</i> Douglas ex. Benth	Hydrophyllaceae	RMH tba	P	Colorado, Delta	8.VI.2011	tba	65.3
<i>Hydrophyllum fendleri</i> (Gray) Heller	Hydrophyllaceae	ID 161100	P	Washington, Yakima	9.VI.2008	tba	1429.7
<i>Hydrophyllum fendleri</i> (Gray) Heller	Hydrophyllaceae	ID 164040	P	Idaho, Idaho	27.V.2009	tba	1014.4
<i>Agastache pallidiflora</i> (Heller) Rydberg	Lamiaceae	CHIC tba	S	Arizona, Coconino	17.VII.2020	tba	617.7
<i>Chamerion angustifolium</i> (L.) Holub	Lamiaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Delphinium barbeyi</i> (Huth) Huth	Ranunculaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Delphinium nuttallianum</i> Pritz.	Ranunculaceae	ID 166162	P	Idaho, Gem	15.VI.2011	tba	9825.5
<i>Delphinium nuttallianum</i> Pritz.	Ranunculaceae	ID 179376	P	Idaho, Gooding	29.IV.2017	tba	733.7
<i>Potentilla fruticosa</i> Pursh	Rosaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Potentilla fruticosa</i> Pursh	Rosaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Potentilla hippiana</i> Lehman.	Rosaceae	CHIC tba	S	New Mexico, Catron	15.VIII.2020	tba	573.8

(Continued on Next Page)

Table 1: samples used in creating the Reference Library (*continued*)

Taxon	Family	Accession	Pres.	Locality	Date Col.	GenBank	Dist. (km)
Potentilla pulcherrima Lehman.	Rosaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6

^a Accession includes both Herbarium and Accession number

^b Pres. refers to Preservation method. 'S' denotes silica gel dried, 'P' denotes pressed

^c All Localities are in the United States of America

666 Appendix 4 - All Pollen Reference Slides Used to Establish Morphotypes

Table 1: All Pollen Voucher Slides Consulted

Taxon	Family	Locality	Accession	Type	Prepared by	Date
<i>Cymopterus lemnoides</i> (J.M. Coulter & Rose) Dorn	Apiaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Heracleum sphondylium</i> L.	Apiaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Ligusticum porteri</i> J.M. Coulter & Rose	Apiaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Osmorhiza depauperata</i> Phil.	Apiaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Maianthemum stellatum</i> (L.) Link	Asparagaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Achillea millefolium</i> L.	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Achillea millefolium</i> L.	Asteraceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Acourtia wrightii</i> (A. Gray) Reveal & King	Asteraceae	NV, Clark, Gold Butte	tba	Novo	E.J.W.	2021
<i>Antennaria racemosissima</i> Hook.	Asteraceae	WY, Park, Ishawooa Mesa	tba	Novo	E.J.W.	2021
<i>Arnica latifolia</i> Bong.	Asteraceae	ID, Blaine, Wildhorse Canyon	tba	Novo	E.J.W.	2021
<i>Artemisia scopulorum</i> A. Gray	Asteraceae	CO, Hinsdale, Uncompahgre Peak	tba	Novo	E.J.W.	2021
<i>Canadanthus modestus</i> (Lindl.) G.L. Nesom	Asteraceae	ID, Idaho, Whiskey Creek	tba	Novo	E.J.W.	2021
<i>Chaenactis douglasii</i> (Hook.) Hook. & Arn.	Asteraceae	MT, Carbon, Pryor Mtn. Rd.	tba	Novo	E.J.W.	2021
<i>Erigeron corymbosus</i> Nutt.	Asteraceae	MT, Carbon, Pryor Mtn. Rd.	tba	Novo	E.J.W.	2021
<i>Erigeron flagellaris</i> A. Gray	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Erigeron speciosus</i> (Lind.) DC.	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Erigeron speciosus</i> (Lindl.) DC.	Asteraceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Helianthella quinquenervis</i> (Hook.) A. Gray	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Heliotropium multiflorum</i> Nutt.	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Heliotropium multiflorum</i> Nutt.	Asteraceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Heterotheca villosa</i> (Pursh) Shinners	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Heterotheca villosa</i> (Pursh) Shinners	Asteraceae	AZ, Coconino, Lake Mary Rd. & 209	tba	Novo	E.J.W.	2021
<i>Hymenoxys hoopesii</i> (A. Gray) Bierner	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Hymenoxys rusbyi</i> (A. Gray) Cockerell	Asteraceae	AZ, Coconino, Lake Mary Rd. & 209	tba	Novo	E.J.W.	2021
<i>Ionactis stenomeria</i> (A. Gray) Greene	Asteraceae	ID, Idaho, Marshall Mountains	tba	Novo	E.J.W.	2021
<i>Senecio hydrophilus</i> Nutt.	Asteraceae	ID, Custer, E. fl. Salmon River	tba	Novo	E.J.W.	2021
<i>Senecio integrerrimus</i> Nutt.	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Senecio serrula</i> Hook.	Asteraceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Senecio wootonii</i> Greene	Asteraceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Solidago lepida</i> DC.	Asteraceae	ID, Idaho, American River	tba	Novo	E.J.W.	2021
<i>Symphotrichum foliacum</i> (Lindl. ex DC.) G.L. Nesom	Asteraceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Symphotrichum subspicatum</i> (Nees) G.L. Nesom	Asteraceae	ID, Custer, E. fl. Salmon River	tba	Novo	E.J.W.	2021
<i>Taraxacum officinale</i> F.H. Wigg	Asteraceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Taraxacum officinale</i> F.H. Wigg	Asteraceae	IL, McHenry, Barrington	tba	Novo	E.J.W.	2021
<i>Lappula squarrosa</i> (Retz.) Dumort.	Boraginaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Mertensia ciliata</i> (James ex Torr.) G. Don	Boraginaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Mertensia fusiformis</i> Greene	Boraginaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Boechera</i>	Brassicaceae	NV, Washoe, Mt. Rose	tba	Novo	E.J.W.	2021
<i>Boechera stricta</i> (Graham) Al-Shehbaz	Brassicaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Cardamine cordifolia</i> A. Gray	Brassicaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Draba aurea</i> Vahl. Ex Hornem	Brassicaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014

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Table 1: All Pollen Voucher Slides Consulted (*continued*)

Taxon	Family	Locality	Accession	Type	Prepared by	Date
<i>Draba spectabilis</i> Greene	Brassicaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Erysimum capitatum</i> (Douglas ex Hook.) Greene	Brassicaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Lepidium montanum</i> Nutt.	Brassicaceae	NM, Catron, Zuni Dry Lake	tba	Novo	E.J.W.	2021
<i>Smelowskia americana</i> Rydb.	Brassicaceae	ID, Blaine, Pioneer Mtns Crest	tba	Novo	E.J.W.	2021
<i>Thlaspi arvense</i> L.	Brassicaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Campanula rotundifolia</i> L.	Campanulaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Campanula rotundifolia</i> L.	Campanulaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Downingia</i>	Campanulaceae	CA, Nevada, Truckee Meadows	tba	Novo	E.J.W.	2021
<i>Lonicera involucrata</i> (Richardson) Banks ex Spreng.	Caprifoliaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Minuartia nuttallii</i> (Pax.) Briq.	Caryophyllaceae	ID, Blaine, Wildhorse Canyon	tba	Novo	E.J.W.	2021
<i>Stellaria longifolia</i> Muh. Ex. Willd.	Caryophyllaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Parnassia kotzebuei</i> Cham. ex Spreng	Celastraceae	ID, Lemhi, Terrace Lakes	tba	Novo	E.J.W.	2021
<i>Sedum lanceolatum</i> Torr.	Crassulaceae	ID, Lemhi, Terrace Lakes	tba	Novo	E.J.W.	2021
<i>Astragalus robbinsii</i> (Oakes) A. Gray	Fabaceae	ID, Custer, Lake Creek	tba	Novo	E.J.W.	2021
<i>Glycyrrhiza lepidota</i> Nutt.	Fabaceae	ID, Butte, Warm Springs Creek	tba	Novo	E.J.W.	2021
<i>Lathyrus eucomus</i> Butters & H. St. John	Fabaceae	NM, Catron, Zuni Salt Lake	tba	Novo	E.J.W.	2021
<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i> (Rydb.) Dorn	Fabaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Lathyrus lanszwertii</i> var. <i>leucanthus</i> (Rydb.) Dorn	Fabaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Lupinus argenteus</i> Pursh	Fabaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Lupinus argenteus</i> Pursh	Fabaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Lupinus argenteus</i> Pursh	Fabaceae	NV, Pershing, Star Peak Canyon	tba	Novo	E.J.W.	2021
<i>Lupinus crassus</i> Payson	Fabaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Lupinus sericeus</i> Pursh	Fabaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Melilotus albus</i> Medik.	Fabaceae	NM, Catron, Hwy 159	tba	Novo	E.J.W.	2021
<i>Trifolium hybridum</i> L.	Fabaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Trifolium pratense</i> L.	Fabaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Vicia americana</i> Muhl. Ex Willd.	Fabaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Vicia americana</i> Muhl. Ex Willd.	Fabaceae	UT, Cache, Spawn Creek	tba	Novo	E.J.W.	2021
<i>Vicia americana</i> Muhl. Ex Willd.	Fabaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Geranium</i>	Geraniaceae	NM, Catron, Jim Smith TH. Rd.	tba	Novo	E.J.W.	2021
<i>Geranium richardsonii</i> Fisch. Trautv.	Geraniaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Phacelia</i> sp.	Hydrophyllaceae	NV, Nye, Toiyabe Crest	tba	Novo	E.J.W.	2021
<i>Iris missouriensis</i> Nutt.	Iridaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Agastache palliflora</i> (A. Heller) Rydb.	Lamiaceae	AZ, Coconino, Lake Mary Rd. & 209	tba	Novo	E.J.W.	2021
<i>Erythronium grandiflorum</i> Pursh	Liliaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Linum lewisii</i> Pursh	Linaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Zigadenus elegans</i> Pursh	Melanthiaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Anticlea elegans</i> (A. Gray) Zomlefer & Judd	Melanthiaceae	ID, Blaine, Wildhorse Canyon	tba	Novo	E.J.W.	2021
<i>Cistanthe/Calyptidium</i>	Montiaceae	NV, Nye, Toiyabe Crest	tba	Novo	E.J.W.	2021
<i>Chamerion angustifolium</i> (L.) Holub	Onagraceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Epilobium obcordatum</i> A. Gray	Onagraceae	ID, Lemhi, Bighorn Crags	tba	Novo	E.J.W.	2021
<i>Castilleja miniata</i> Douglas ex Hook.	Orobanchaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010

(Continued on Next Page)

Table 1: All Pollen Voucher Slides Consulted (*continued*)

Taxon	Family	Locality	Accession	Type	Prepared by	Date
<i>Castilleja sulphurea</i> Rydb.	Orobanchaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Pedicularis groenlandica</i> Retz.	Orobanchaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Pedicularis racemosa</i> Douglas ex Benth.	Orobanchaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Corydalis aurea</i> Willd.	Papaveraceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Erythranthe guttata</i> (DC.) G.L. Nesom	Phrymaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Penstemon wilcoxii</i> Rydb.	Plantaginaceae	MT, Missoula, Mission Mtns vic.	tba	Novo	E.J.W.	2021
<i>Collomia linearis</i> Nutt.	Polemoniaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Phlox condensata</i> (A. Gray) E.E. Nelson	Polemoniaceae	CO, Hinsdale, Uncompahgre Peak	tba	Novo	E.J.W.	2021
<i>Polemonium foliosissimum</i> A. Gray	Polemoniaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Polemonium occidentale</i> Greene	Polemoniaceae	ID, Custer, Lake Creek	tba	Novo	E.J.W.	2021
<i>Polemonium viscosum</i> Nutt.	Polemoniaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2010
<i>Bistorta bistortoides</i> (Pursh) Small	Polygonaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Eriogonum</i> spp.	Polygonaceae	NV, Washoe, Hwy 445	tba	Novo	E.J.W.	2021
<i>Polygala barbeyana</i> Chodat	Polygonaceae	NM, Eddy, Yeso Hills	tba	Novo	E.J.W.	2021
<i>Polygonum polygaloides</i> L.	Polygonaceae	MT, Missoula, Mission Mtns	tba	Novo	E.J.W.	2021
<i>Androsace filiformis</i> Retz.	Primulaceae	ID, Custer, Bradshaw Creek	tba	Novo	E.J.W.	2021
<i>Aquilegia coerulea</i> E. James	Ranunculaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Aquilegia coerulea</i> E. James	Ranunculaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Aquilegia elegantula</i> Greene	Ranunculaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Delphinium barbeyi</i> (Huth) Huth	Ranunculaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Delphinium nuttallianum</i> Pritz. Ex Walp.	Ranunculaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Ranunculus alismifolius</i> Geyer ex Benth.	Ranunculaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Ranunculus glaberrimus</i> Hook.	Ranunculaceae	ID, Lemhi, Agency Creek	tba	Novo	E.J.W.	2021
<i>Ranunculus inamoenus</i> Greene	Ranunculaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Ranunculus</i> spp.	Ranunculaceae	NV, Washoe, Mt. Rose	tba	Novo	E.J.W.	2021
<i>Thalictrum sparsiflorum</i> Tuzc. Ex Fisch. & C.A. Mey.	Ranunculaceae	ID, Custer, E. fk. Salmon River	tba	Novo	E.J.W.	2021
<i>Dasiphora fruticosa</i> (L.) Rydb.	Rosaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Dasiphora fruticosa</i> (L.) Rydb.	Rosaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Fragaria virginiana</i> Duchesne	Rosaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Geum triflorum</i> Pursh	Rosaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Potentilla biennis</i> Greene	Rosaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Potentilla hippiana</i> Lehm.	Rosaceae	NM, Catron, Jim Smith TH.Rd.	tba	Novo	E.J.W.	2021
<i>Potentilla pulcherrima</i> Lehm.	Rosaceae	CO, Gunnison, Gothic	None	Physical	P.J.C.	2014
<i>Potentilla pulcherrima</i> Lehm.	Rosaceae	CO, Gunnison, Washington Gulch	tba	Novo	E.J.W.	2021
<i>Salix bebbiana</i> Sarg.	Salicaceae	ID, Custer, Lake Creek	tba	Novo	E.J.W.	2021
<i>Salix geyeriana</i> Andersson	Salicaceae	ID, Butte, Clyde	tba	Novo	E.J.W.	2021
<i>Mitella stauropetala</i> Piper	Saxifragaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Valeriana occidentalis</i> A. Heller	Valerianaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010
<i>Viola canadensis</i> L.	Violaceae	CO, Gunnison, Gothic	None	Image	H.M.B.	2010

* All Localities are in the United States of America

† Accession refers to whole-plant vouchers, all specimens are deposited at CHIC.

‡ Type Refers to whether both a physical and digital copy exist; 'Image' denotes only digital

§ Date refers to the Date of preparation.

670 POLLEN CLUSTER RESULTS SHOULD BE HERE

- 1a: Pollen shed in clumps (tetrads/polyads); grains generally triangular, with an annulus subtending the porate apertures (go 34)
- 1b: Pollen generally dispersed as single units (monads); grains seldom if ever with annulus.
- 2a: Apertures porate, always lacking colpi
- 3a: grain outline from equatorial view circular
- 4a: Pores distributed along the equator.
- 5a: Pores > 5 (stephanoporate)
- 6a: Ornamentation homobrochate (~ *MENTZELIA*)
- 6b: Ornamentation otherwise (~ *POLYGALA*)
- 5b: Pores < 5 (*CURRENTLY OPEN*)
- 4b: Pores +/- distributed across grain (pantoporate)
- 7a: Ornamentation with striate ornamentation (~ *POLEMONIUM*)
- 7b: Ornamentation otherwise
- 8a: Ornamentation, slightly irregular - without regularly repeating features (scabrate) (~ *STELLARIA*)
- 8b: Ornamentation forming regularly repeating (reticulate) cells of varying shapes.
- 9a: spacing between the grid cells large (lophate), the walls of the cells with another set of projecting ornamentation (~ *OPUNTIA*)
- 9b: spacing between cells small, the wall of the cells without projecting features.
- 10a: Pores extending beyond the reticulate grids (~ *ARENARIA*)
- 10b: Pores extending beyond the reticulate grids (~ *PHLOX*)
- 3b: Outline from equatorial view otherwise (usually slightly triangular)
- 11a: Outline elliptic (*CURRENTLY EMPTY*)
- 11b: Outline not elliptic, grains often with acute, if rounded, angles along sides (e.g., triangular, polygonal) (*EMPTY*)
- 2b: Apertures with colpi, occasionally also with pores in addition (coporate)
- 12a: Grains with bristles tapering to points (echinate), and tri-colporate.
- 13a: Grains uniformly echinate, less the apertures. (Asteraceae 1)
- 13b: Grains with echinate bristles on ridges of lophae (Asteraceae 2)
- 12b: Grains without echinate ornamentation - this lead includes projections with ornamentation with round tips.
- 14a: Grains with either less than 3 apertures, or with two distinct ornamentation types (generally $\frac{1}{2}$ psilate, $\frac{1}{2}$ reticulate).
- 15a. Grains apparently lacking any apertures. (~ *IRIS*)
- 15b. Grains aperturate
- 16a. Ornamentation on one face of grain psilate, the other homobrochate (~ *ZIGADENUS + ANTICLEA*)
- 16b. Ornamentation psilate across both faces of grain (~*ERYTHONIUM*)
- 14b. Grains with either 3 or more apertures, or with an elongated spiral like aperture
- 17a. Grain with spiral like colpi
- 18a. Spiral with deep well-defined furrows (~ *ERYTHRANTHE GUTTATA*, syn. obsolete. *MIMULUS*)
- 18b. Spirals without well-defined grooves, ornamentation evidently perforate (~ *RANUNCULUS ALISMIFOLIUS*)
- 17b. Grains with colpi these not forming irregular spiral motifs.
- 19a. Grains elliptic, essentially perfectly cylindrical along longest axis, except for minor inundations along equatorial region. Apertures, of two types (heteroaperturate). (~ *BORAGINACEAE*)
- 19b Grains shaped similar or not, but never heteroaperturate.
- 20a. From a polar view, grains notably polygonal (hexagonal), also evident when seldom seen from a equatorial view. (~ *PHACELIA*/ maybe *Hydrophyllaceae*, *Hydrophyllum* not sampled)
- 20b. From a polar view, grains not with 6 convex apices

- 21a. Grains elliptic, with a short colporate aperture on each psilate face, the edges of each face and the apices with a distinct (homobrochate) textured ornamentation. (~ POLYGONUM)
- 21b. Grains otherwise, not featuring a mix of ornamentations independent of the apertures.
- 22a. Ornamentation perforate, the three colpi very short, their longest axis parallel to the equator rather than perpendicular. These colpi often times almost appearing to be slightly raised on an annulus like feature (~ LONICERA)
- 22b. Grains not as described in all aspects of the above.
- 23a. Apertures colporate
- 24a. Outline of grain in equatorial view circular, ornamentation smooth. (~MORPHOTYPE A).
- 24b. Grains otherwise
 - 25a Grains distinctly triangular from polar view (go 26)
 - 25b Grains elliptic (go 27)
 - 26a Grains very large, clearly strongly triangular in cross section. (~ GERANIUM)
 - 26b Grains smaller (SIZE), weakly triangular in cross section (~ POTENTILLA/DASIPHORA in part)
 - 27a Grains elliptic to weakly circular (~MORPHOTYPE B)
 - 27b Grains elliptic, much longer pole to pole than across equator.
 - 28a Grains with evident protrusions of the pore, colpi short, scarcely noticeable (~ APIACEAE)
- 23b. Apertures colpate
 - 30a Ornamentation psilate (~MORPHOTYPE C).
 - 30b Ornamentation otherwise
 - 31a Ornamentation homobrochate (~ MORPHOTYPE D)
 - 31b Ornamentation otherwise
 - 32a. Ornamentation bacculate, grains large, (~LINUM)
 - 32b. Ornamentation otherwise
 - 33a. Ornamentation of minor cross-corrugated grooves (fossulate) (~CORYDALIS)
 - 33b. Ornamentation of scarcely perceptible irregular features (scabrate) (~RANUNCULUS IN PART)

34a: Annula subtending the apertures – making grains appear more or less triangular; Pollen often with viscin threads (ONAGRACEAE)

34b: Apertures not annulate – grains appear more or less circular (~ERICACEAE)

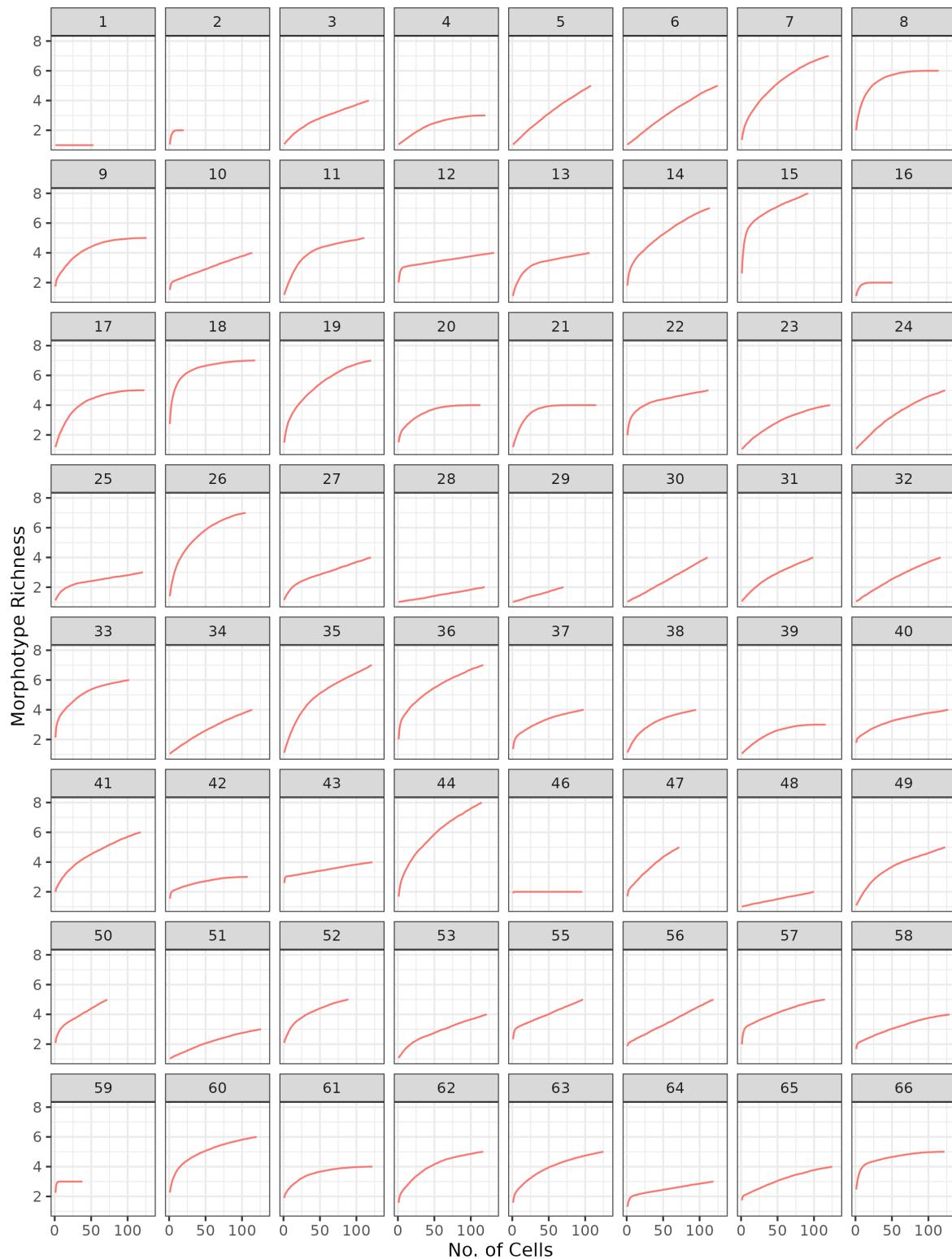
Morphotype A: Trifolium, Lupinus, Glycrrhiza, Mitella, Geum

Morphotype B: Lupinus, Lathyrus, Potentilla, Androsace, Bistorta, Vicia

Morphotype C: Jeffersonia, Micranthes, Prunus, Delphinium, Androsace, Penstemon, Orthocarpus, Scutellaria, Aquilegia, Castilleja, Draba)

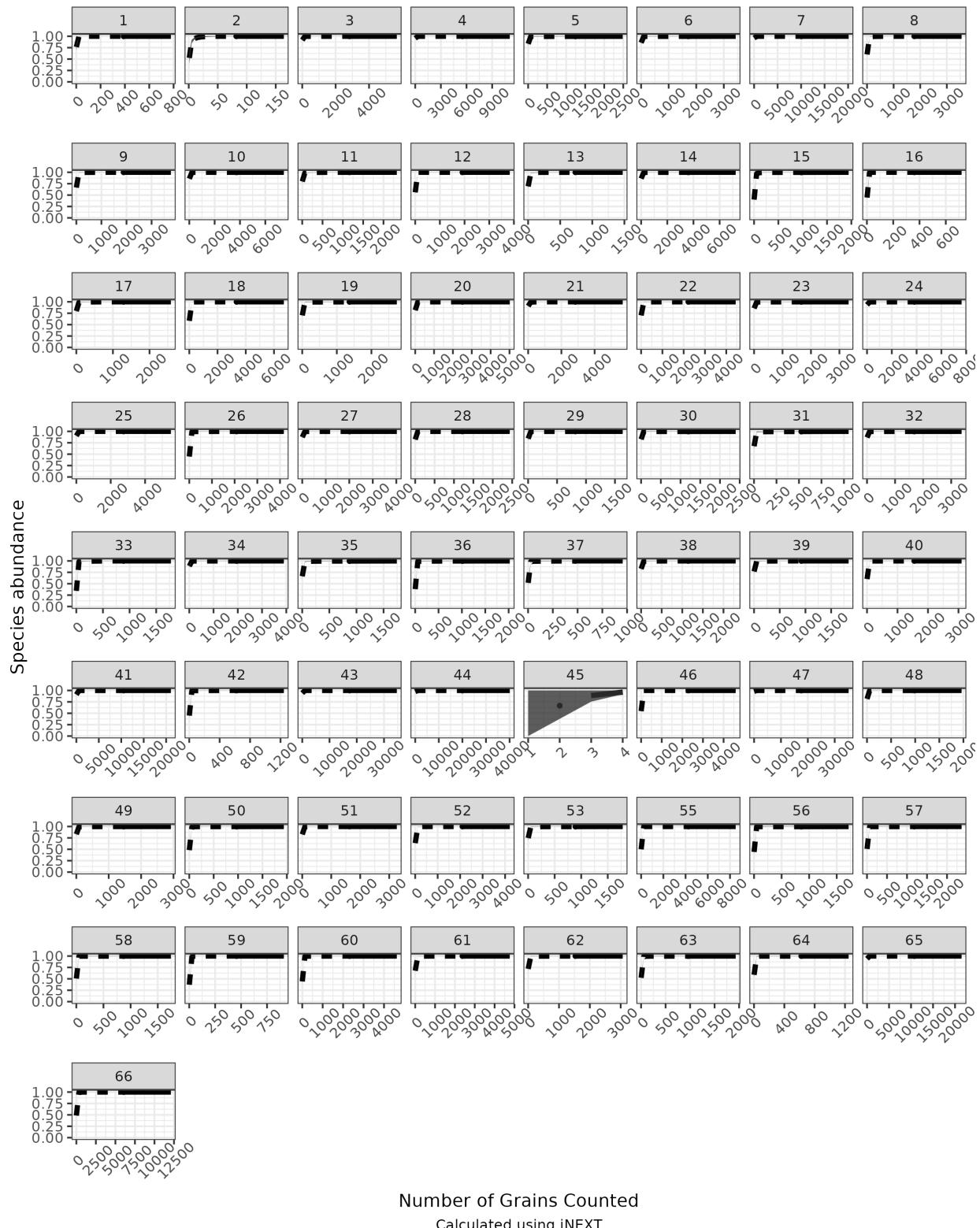
Morphotype D: Salix, Boechera

Rarefaction Curves of Species Richness
Calculated as random plot order with 1000 bootstrap replicates



Used to assess completeness of subsampling. VEGAN package 'specaccum' function used

Species Richness Abundance Estimating via Hill Numbers ($q = 0$)
Confidence Interval of 99% with 1000 Bootstrap replicates



Number of Grains Counted
Calculated using iNEXT

Table 1: All species present in the Reference Sequence Databases
(Kraken and BLAST)

Order	Family	Taxon
Alismatales	Potamogetonaceae	<i>Potamogeton wrightii</i>
Apiales	Apiaceae	<i>Osmorhiza aristata</i>
Asparagales	Amaryllidaceae	<i>Allium stamineum</i>
	Asparagaceae	<i>Streptopus amplexifolius</i>
Asterales	Asteraceae	<i>Anaphalis margaritacea</i> <i>Antennaria carpatica</i> <i>Antennaria dioica</i> <i>Artemisia sibirica</i> <i>Brickellia dentata</i> <i>Chrysanthemus greenei</i> <i>Cirsium pannonicum</i> <i>Cirsium parryi</i> <i>Cirsium vulgare</i> <i>Crepis pygmaea</i> <i>Ericameria parryi</i> <i>Erigeron ecuadorensis</i> <i>Erigeron grandiflorus</i> <i>Erigeron rosulatus</i> <i>Erigeron uniflorus</i> <i>Helianthella quinquenervis</i> <i>Heterotheca villosa</i> <i>Hieracium avilae</i> <i>Hieracium jubatum</i> <i>Hymenoxys hoopesii</i> <i>Leucanthemum graminifolium</i> <i>Microseris lindleyi</i> <i>Omalotheca supina</i> <i>Packera quercetorum</i> <i>Pseudognaphalium attenuatum</i> <i>Pseudognaphalium frigidum</i> <i>Pseudognaphalium lacteum</i> <i>Pseudognaphalium oxyphyllum</i> <i>Rudbeckia hirta</i> <i>Scabrethia scabra</i> <i>Senecio adenophyllus</i> <i>Senecio algens</i> <i>Senecio apolobambensis</i> <i>Senecio candollei</i> <i>Senecio chionogeton</i> <i>Senecio formosus</i> <i>Senecio funcii</i> <i>Senecio gilliesii</i> <i>Senecio humillimus</i> <i>Senecio nutans</i> <i>Senecio puchei</i> <i>Senecio rufescens</i> <i>Senecio spinosus</i> <i>Senecio tephrosioides</i>

(Continued on Next Page)

Table 1: All species present in the Reference Sequence Databases
(Kraken and BLAST) (*continued*)

Order	Family	Taxon
Boraginales	Campanulaceae	<i>Solidago chilensis</i> <i>Stilpnolepis intricata</i> <i>Symphyotrichum foliaceum</i> <i>Taraxacum cucullatum</i> <i>Taraxacum officinale</i>
		<i>Tonestus lyallii</i>
		<i>Townsendia formosa</i>
		<i>Campanula argaea</i>
		<i>Campanula rotundifolia</i>
	Hydrophyllaceae	<i>Cynoglossum amplifolium</i> <i>Cynoglossum anchusoides</i> <i>Cynoglossum pringlei</i> <i>Mertensia ciliata</i> <i>Mertensia fusiformis</i>
		<i>Hydrophyllum canadense</i>
		<i>Hydrophyllum capitatum</i>
		<i>Hydrophyllum fendleri</i>
		<i>Nemophila menziesii</i>
Caryophyllales	Caryophyllaceae	<i>Arenaria globiflora</i> <i>Arenaria serpyllifolia</i> <i>Cerastium arvense</i> <i>Cerastium lanceolatum</i> <i>Minuartia recurva</i> <i>Odontostemma leucasterium</i> <i>Pseudostellaria heterophylla</i> <i>Sagina procumbens</i> <i>Schizotechium monospermum</i> <i>Shivparvatia glanduligera</i>
		<i>Stellaria graminea</i>
		<i>Stellaria holostea</i>
		<i>Stellaria obtusa</i>
		<i>Rumex induratus</i>
		<i>Rumex spinosus</i>
		<i>Parnassia faberi</i>
		<i>Parnassia palustris</i>
		<i>Paxistima canbyi</i>
		<i>Gaultheria prostrata</i>
Celastrales	Ericaceae	<i>Moneses uniflora</i> <i>Orthilia secunda</i> <i>Vaccinium vitis-idaea</i> <i>Collomia grandiflora</i> <i>Ipomopsis aggregata</i>
		<i>Phlox douglasii</i>
		<i>Primulaceae</i>
		<i>Androsace studiosorum</i>
		<i>Androsace vitaliana</i>
	Fabaceae	<i>Astragalus pelecinus</i>
		<i>Lupinus argenteus</i>
		<i>Lupinus sericeus</i>

(Continued on Next Page)

Table 1: All species present in the Reference Sequence Databases (Kraken and BLAST) (*continued*)

Order	Family	Taxon
Gentianales	Gentianaceae	<i>Vicia americana</i> <i>Frasera speciosa</i> <i>Gentiana cruciata</i>
Hyphomicrobiales	Xanthobacteraceae	<i>Azorhizobium caulinodans</i>
Lamiales	Lamiaceae	<i>Agastache pallidiflora</i>
Liliales	Colchicaceae	<i>Prosartes smithii</i>
	Liliaceae	<i>Erythronium dens-canis</i>
	Melanthiaceae	<i>Anticlea elegans</i> <i>Veratrum viride</i>
Malpighiales	Hypericaceae	<i>Hypericum perforatum</i>
	Salicaceae	<i>Populus alba</i>
	Violaceae	<i>Viola odorata</i>
Myrtales	Onagraceae	<i>Chamaenerion angustifolium</i> <i>Epilobium canum</i> <i>Epilobium parviflorum</i>
Ranunculales	Berberidaceae	<i>Berberis sibirica</i>
	Papaveraceae	<i>Corydalis aitchisonii</i>
	Ranunculaceae	<i>Actaea heracleifolia</i> <i>Anemone anemonoides</i> <i>Anemone obtusiloba</i> <i>Aquilegia ecalcarata</i> <i>Caltha palustris</i> <i>Delphinium barbeyi</i> <i>Delphinium gracile</i> <i>Delphinium nuttallianum</i> <i>Pulsatilla chinensis</i> <i>Thalictrum thalictroides</i> <i>Thalictrum tuberosum</i> <i>Trollius europaeus</i>
Rosales	Elaeagnaceae	<i>Shepherdia argentea</i>
	Rosaceae	<i>Crataegus bipinnatifida</i> <i>Dasiphora fruticosa</i> <i>Geum ternatum</i> <i>Hedlundia austriaca</i> <i>Holodiscus argenteus</i> <i>Karpatiosorbus devoniensis</i> <i>Micromeles japonica</i> <i>Potentilla anserina</i> <i>Potentilla pulcherrima</i> <i>Potentilla tetrandra</i> <i>Rubus chingii</i>
Sapindales	Sapindaceae	<i>Acer campestre</i>
Saxifragales	Crassulaceae	<i>Rhodiola rosea</i> <i>Sedum nudum</i>
	Grossulariaceae	<i>Ribes rubrum</i>
	Saxifragaceae	<i>Lithophragma parviflorum</i> <i>Saxifraga biflora</i> <i>Saxifraga fortunei</i>

(Continued on Next Page)

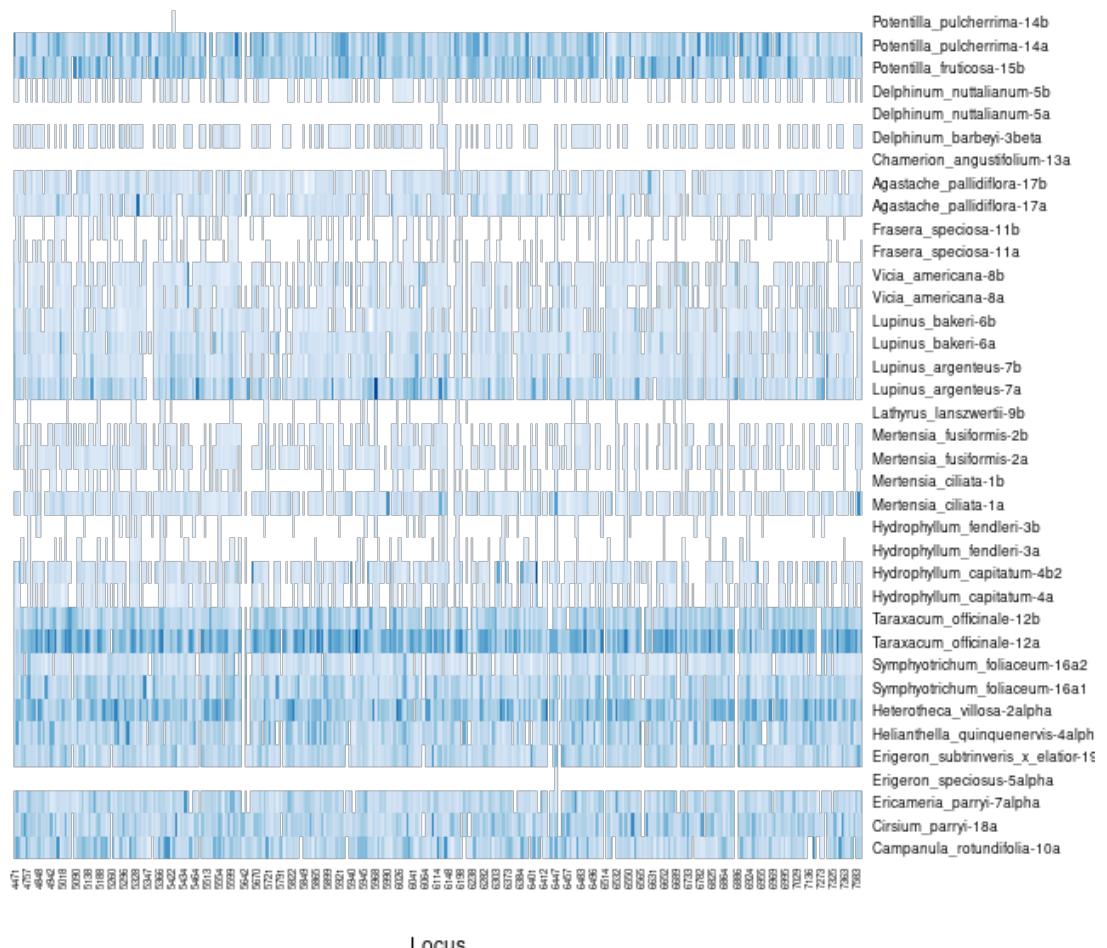
683 Appendix XX - All Species in the Sequence Databases (con't)

Table 1: All species present in the Reference Sequence Databases
(Kraken and BLAST) (*continued*)

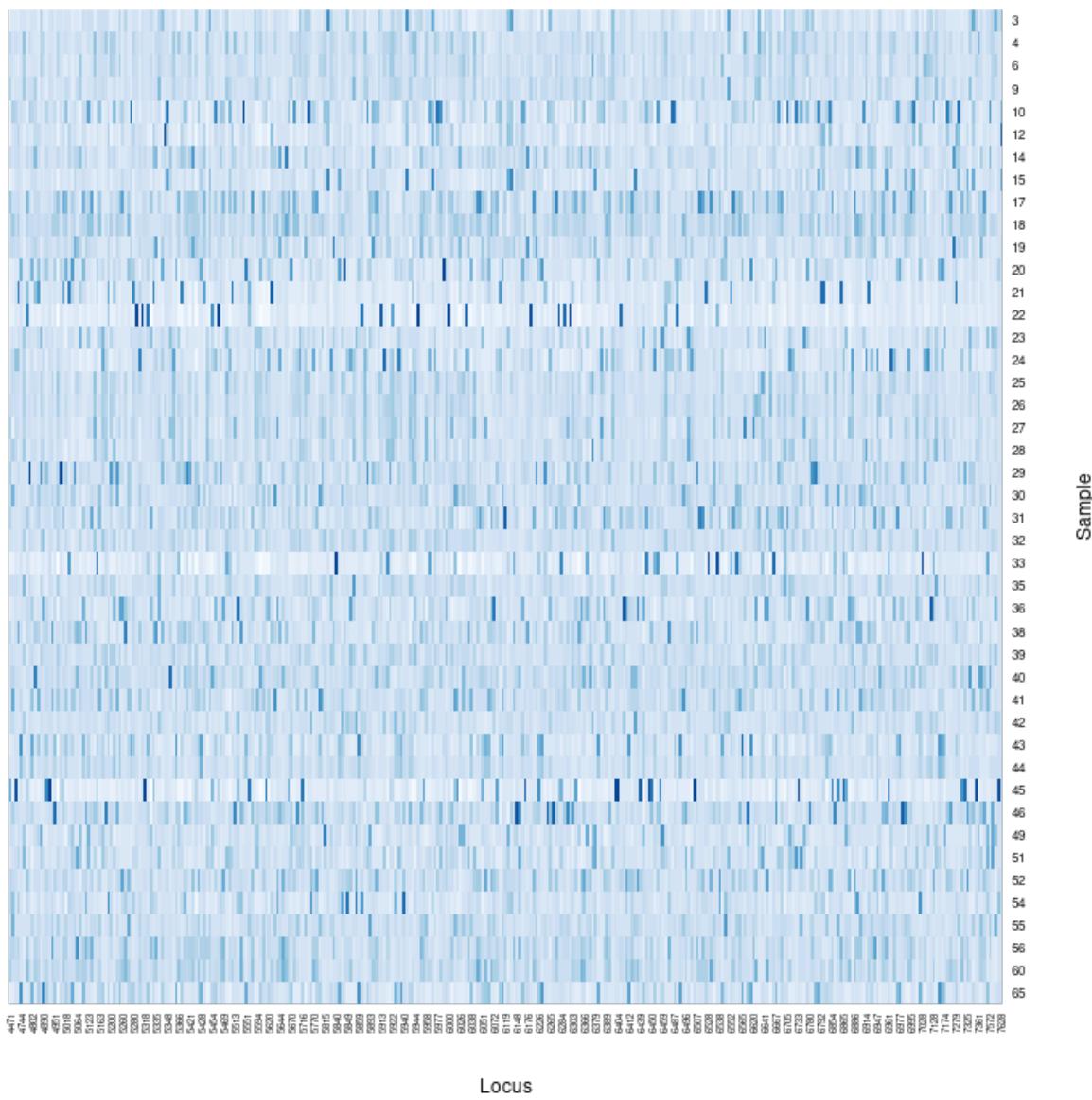
Order	Family	Taxon
		Saxifraga maderensis
		Saxifraga oppositifolia
		Saxifraga portosanctana
		Saxifraga x geum

x geum* \end{longtable}

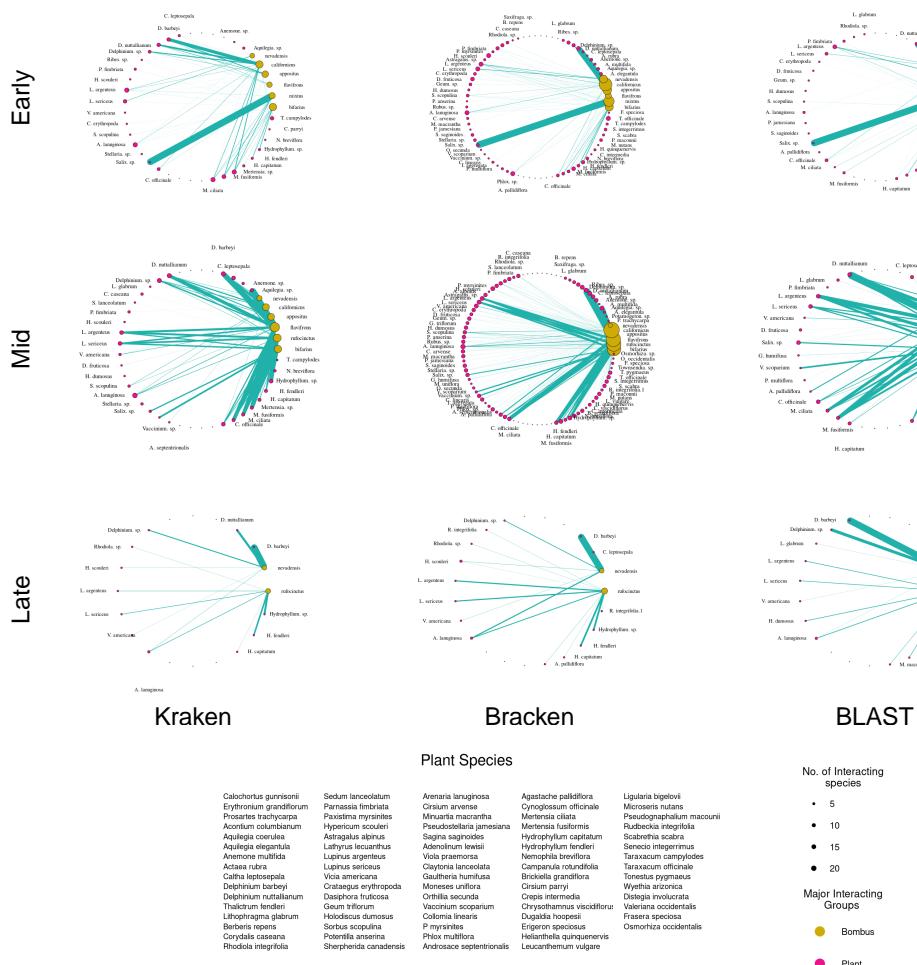
Loci & Nucleotides Returned per Reference Sample



Percent matched reads



Comparision of Foraging Patterns from Three Sequence Alignment Algorithms



690 Appendix XX - Models used for Species Distribution Model Ensembles

691 The two machine learning models utilize Ensemble learning.

692 **Ensemble learning** utilizes many sets of trees, each tree being composed of many binary decisions, to
693 create a single model. Each independent variable (- or *feature*) may become a node on the tree - i.e. a
694 location on the tree where a binary decision will move towards a predicted outcome. Each of the decision
695 tree models which ensemble learning utilizes is a weak model, each of which may suffer due to high variance
696 or bias, but which produce better outcomes than would be expected via chance. When ensembled these
697 models generate a strong model, a model which should have more appropriately balanced variance and bias
698 and predicts outcomes which are more strongly correlated with the expected values than the individual weak
699 models.

700 **Random Forest (RF)** the training data are continually bootstrap re-sampled, in combination with random
701 subsets of features, to create nodes which attempt to optimally predict a known outcome. A large number
702 of trees are then aggregated, via the most common predictions, to generate a final classification prediction
703 tree. Each individual prediction tree is generated independently of the others.

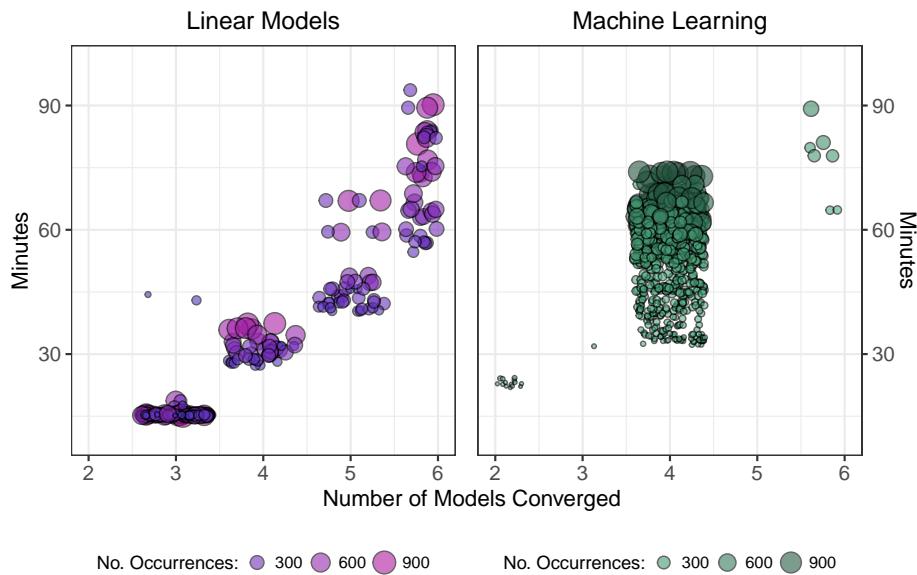
704 **Boosted Regression Tree (BRT)** (or Gradient Boosted tree) An initial tree is grown, and all other trees
705 are derived sequentially from it, as each new tree is grown the errors in responses from the last tree are
706 weighed more heavily so that the model focuses on selecting dependent variables which refine predictions.
707 All response data and predictor variables are kept available to all trees.

708 **Bias** predictions from an algorithm are systematically in error due to being prejudiced for or against certain
709 results, due to assumptions during learning.

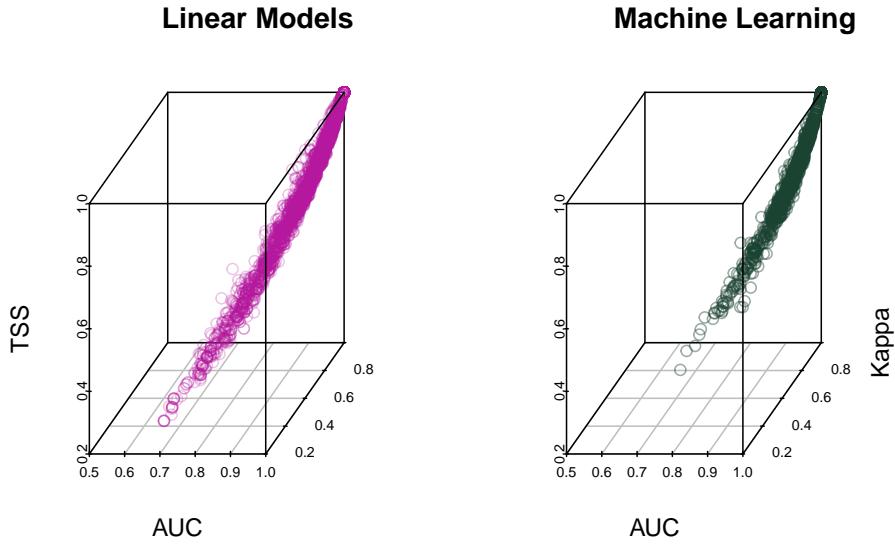
710 **Variance** errors in models due to an over-reliance and sensitivity of training to outliers in training data.

711 In general, Random Forest models have high bias and low variance, where boosted regressions trees have lower
712 bias and higher variance. Theoretically, the weaknesses and strengths of bootstrap aggregation (bagging) as
713 implemented by Random Forests are supplemented by the boosting.

Time Spent Fitting and Projecting Models onto Gridded Surfaces



Collectively it took 215 hours for all of the GLM and GAM to run, and for the converged models to be ensembled, and predicted onto a raster surface; it took 419 hours for the same process to be carried out for the Random Forest and Boosted Regression Tree models.



Results for each converged individual model which were then ensembled, using weights from the True Skill Statistic (TSS).

Table 1: Subset of Possible Combinations for re-classifying Sequences by Incorporating Ecological Factors

Spatial	Temporal	Congener	Confamilial	Congeners	Confamilials	Condition	Return	Rank
1	1	1	1	0	0	A.1	Input	Species
1	1	1	1	1	0	A.2	Input	Species
1	1	1	1	0	1	A.3	Input	Species
1	1	1	1	1	1	A.4	Input	Species
1	1	1	0	0	0	A.5	Input	Species
1	1	1	0	1	0	A.6	Input	Species
1	1	0	1	0	0	A.7	Input	Species
1	1	0	1	0	1	A.8	Input	Species
1	1	0	0	0	0	A.9	Input	Species
1	0	1	1	0	0	B.1	Congener	Species
1	0	1	1	0	1	B.2	Congener	Species
1	0	1	0	0	0	B.3	Congener	Species
1	0	1	1	1	0	C.1	Congener	Genus
1	0	1	1	1	1	C.2	Congener	Genus
1	0	1	0	1	0	C.3	Congener	Genus
1	0	0	1	0	0	D.1	Confamilial	Species
1	0	0	1	0	1	E.1	Confamilial	Family
1	0	0	0	0	0	F.1	Input	Species
0	0	1	1	0	0	G.1	Congener	Species
0	0	1	1	0	1	G.2	Congener	Species
0	0	1	0	0	0	G.3	Congener	Species
0	0	1	1	1	0	H.1	Congener	Genus
0	0	1	1	1	1	H.2	Congener	Genus
0	0	1	0	1	0	H.3	Congener	Genus
0	0	0	1	0	0	I.1	Confamilial	Species
0	0	0	1	0	1	J.1	Confamilial	Family

Note, for both ‘Congener’ and ‘Confamilial’ (*in the singular*) ‘1’ denotes that a species is present; in a sense the genus is monotypic in space and time. For both ‘Congeners’ and ‘Confamilials’ (*in the plural*), ‘1’ denotes that two or more species are present; ‘Confamilial’ again representing a monotypic entity in space and time.

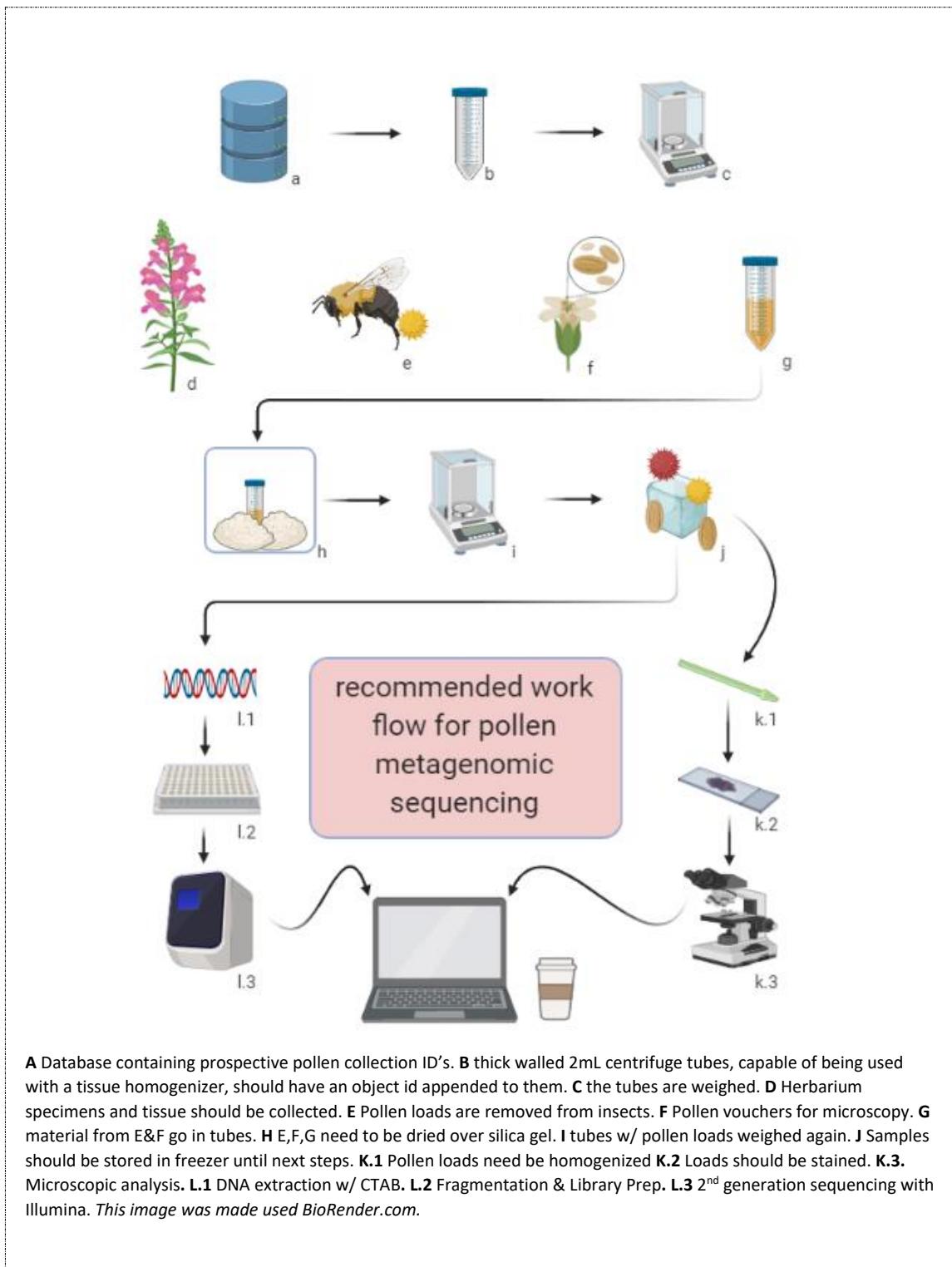
$$\begin{aligned} \text{Spatial} == 1 \& \text{ Temporal} == 1 \sim \mathbf{A} \\ \text{Spatial} == 1 \& \text{ Temporal} == 0 \& \text{ Congener} = 1 \sim \mathbf{B} \end{aligned}$$

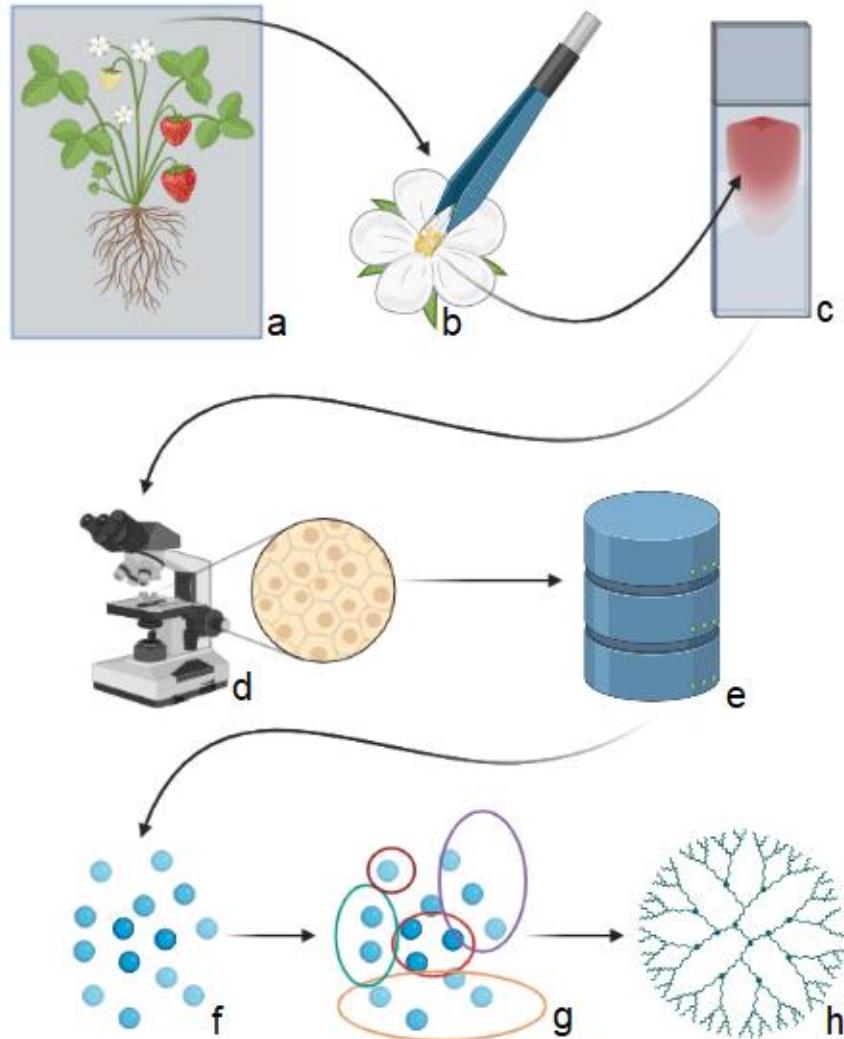
The temporal dimension is now buffered and a form of \mathbf{A} is employed
 $\text{Spatial} == 1 \& \text{ Temporal} +/- \text{ Buffer} == 1 \sim \mathbf{X}$

$$\begin{aligned} \text{Spatial} == 1 \& \text{ Temporal} == 0 \& \text{ Congeners} >= 2 \sim \mathbf{C} \\ \text{Spatial} == 1 \& \text{ Temporal} == 0 \& \text{ Congeners} == 0 \& \text{ Confamilial} == 1 \sim \mathbf{D} \\ \text{Spatial} == 1 \& \text{ Temporal} == 0 \& \text{ Congeners} == 0 \& \text{ Confamilial} >= 2 \sim \mathbf{E} \\ \text{Spatial} == 1 \& \text{ Temporal} == 0 \& \text{ Congener|s} == 0 \& \text{ Confamilial|s} == 0 \sim \mathbf{F} \end{aligned}$$

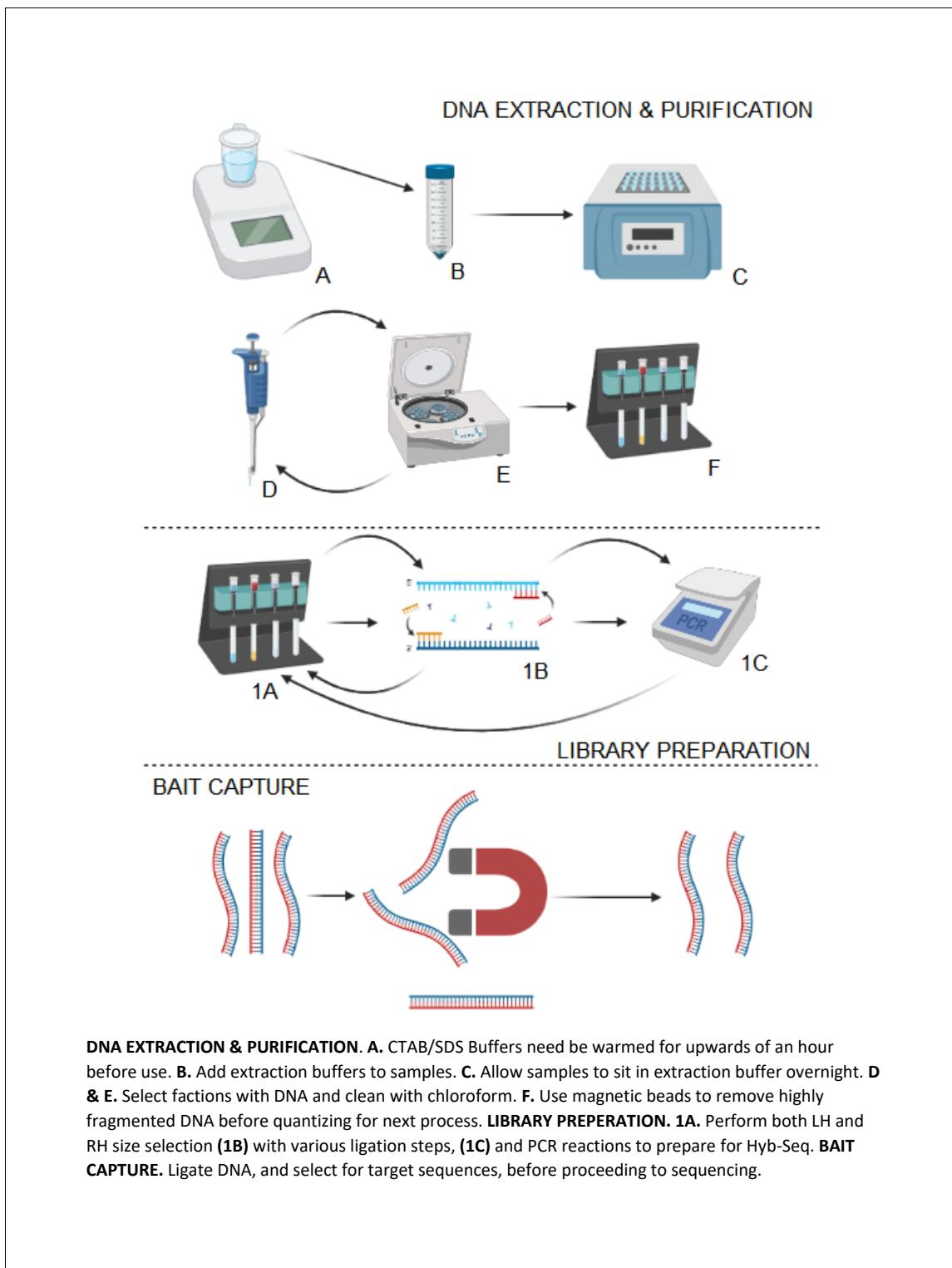
$$\begin{aligned} \text{Spatial} == 0 \& \text{ Temporal} == 0 \& \text{ Congener} == 1 \sim \mathbf{G} \\ \text{Spatial} == 0 \& \text{ Temporal} == 0 \& \text{ Congeners} == 1 \sim \mathbf{H} \\ \text{Spatial} == 0 \& \text{ Temporal} == 0 \& \text{ Confamilial} == 1 \sim \mathbf{I} \\ \text{Spatial} == 0 \& \text{ Temporal} == 0 \& \text{ Confamilials} == 1 \sim \mathbf{J} \end{aligned}$$

While the overall order matters, \mathbf{X} in particular may significantly alter conclusions.





A. An herbarium collection in flower from which pollen may be removed. **B.** The careful removal of pollen from dehiscent anthers using dissection tools. **C.** Place the pollen on a fuchsin jelly cube and melt it with stirring on a hotplate, for ca. 30 seconds. **D.** Microscopic imaging, of specimens and collection of character trait data. **E.** Immediate input and accessioning of files to a database. **F.** Ordination of traits into 2-dimensional space. **G.** Agglomerative clustering of data points into similar groups. **H.** Recovery of bifurcating decisions in development of clusters, or handwritten keys to visually diagnosable groups.



DNA EXTRACTION & PURIFICATION. A. CTAB/SDS Buffers need to be warmed for upwards of an hour before use. B. Add extraction buffers to samples. C. Allow samples to sit in extraction buffer overnight. D & E. Select fractions with DNA and clean with chloroform. F. Use magnetic beads to remove highly fragmented DNA before quantizing for next process. **LIBRARY PREPARATION.** 1A. Perform both LH and RH size selection (1B) with various ligation steps, (1C) and PCR reactions to prepare for Hyb-Seq. **BAIT CAPTURE.** Ligate DNA, and select for target sequences, before proceeding to sequencing.

CTAB-DNA POLLEN EXTRACTIONS

Adapted from Lalhmangiahi et. al & Guertler et al. by Benkendorf, Fant, & Noble.

SAMPLE PREPARATION AND GRINDING

- a1) Add 380 μ L extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5). This solution will need to be warm enough for the SDS to be in solution, requires heat and stirring.
- a2) Vortex samples at speed > 2000, until pellet breaks apart, ca. 20-30 seconds.
- a3) Use the Pellet Pestle Motor (Kontes) for ca. 15 seconds to macerate samples.
- a4) Add 100 μ L extraction buffer to wash the tip of the pestle into the centrifuge tube, and burst bubbles.
- a5) Allow to sit at 35°C for 1 hour, use vortex occasionally if sedimentation of pollen occurs.

EXTRACTION AND ISOLATION OF DNA

- b1) Warm CTAB buffer to remove any precipitants if present.
- b2) Add 480 μ L 10% CTAB buffer.
- b3) Add 10 μ L RNase (10mg/mL); invert by hand, incubate for 40 minutes at 37°C, increase heat to 60°C wait 20 minutes before continuing to b4.
- b4) Add 15 μ L proteinase K (20mg/mL) & 12.5 μ L DTT (1 molar in H₂O); invert by hand, incubate for 1 hr. at 60°C.
- b5) Incubate overnight at 40°C (*note: this is a hard stopping point*)
- b6) Add 500 μ L of Phenol-chloroform-isoamyl alcohol vortex samples, centrifuge at 10,000 rpm (10 min.)
- b7) Transfer the uppermost aqueous layer to a new 2 mL centrifuge tube.

DNA PRECIPITATION

- c1) Add slightly chilled Isopropyl alcohol & Sodium Acetate 3mM 5:1, equivalent to ca. 2/3 of the removed layer. Store at -20°C, 1 hour to allow precipitation.
(Note: potential stopping point for a day or more, samples can stay at -20°C for days)
- c2) Centrifuge at 13,000 rpm for 10 minutes.
- c3) Pour supernatant into new 2mL centrifuge tube, add 400 μ L 70% EtOH. Store at -20°C for 20 minutes.
- c4) Spin at 13,000 rpm for 10 minutes, discard supernatant.
for both tubes the following steps apply
- c3) Add 400 μ L of 75% EtOH, invert tube x3, centrifuge at 13,000 rpm for 4 minutes; discard supernatant
- c4) Add 400 μ L of 95% EtOH, invert tube x3, centrifuge at 13,000 rpm for 4 minutes, discard supernatant
- c5) Dry tubes in vacuum centrifuge for 30 minutes on medium heat at 15 mmHG.

RESUSPENSION OF DNA

- d1) Add 40 μ L of dna free H₂O to sample.
- d2) place on heat block at 37°C until pellet resuspends with occasional use of vortexes.

NOTES: a 10% CTAB preparation will not readily stay in solution, maintain it on heatblock until you are ready to use it. After adding it to extraction tubes move them to heat block immediately (*i.e.* in batches of 5-10).

Solutions

Extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 50 mM NaCl, 10% SDS - pH 7.5, ca. 32 mL H₂O)

For 100 samples (50 mL solution)

10 grams SDS (Sodium Dodecyl Sulfate , d = 1.01 g/cm3)

146.1 mg Sodium Chloride (NaCl, mw = 58.4 g/mol)

930.6 mg EDTA (EthyleneDiamineTetraacetic Acid Disodium Salt dihydrate, mw = 372.24 g/mol)

Add 20 mL deH₂O

5 mL Tris-HCl pH 8.0 (1 molar- kept in fridge)

Fill to 50 mL with deH₂O

Auto clave on 'Liquid' setting for 15 minutes.

Dissolution may require heat and stirring (3 & 4 settings respectively, ca. 15 min.)

10% CTAB solution (20 mM Tris-Cl pH 8.0, 1.4 M NaCl, 10 mM EDTA pH 7.5, 10% CTAB, 5% PVP, 40 mL DiH₂O)

For 100 samples (50 mL solution)

add ~30 mL deH₂O,

1 ml Tris-HCl pH 8.0 (1 molar- kept in fridge; 2-Amino-2-(hydroxymethyl)propane-1,3-diol)

4.08 g Sodium Chloride (NaCl, mw = 58.4 g/mol)

4 mL EDTA pH 7.5 (0.125 molar – kept in fridge; 2,2',2'',2'''-(Ethane-1,2-diyl)dinitrilo)tetraacetic acid)

5 g CTAB (hexadecyl(trimethyl)ammonium bromide, mw = 364.45, FYI this is 274 mM)

Auto clave on 'Liquid' setting for 15 minutes.

2.5 g PVP-40 (1-ethenylpyrrolidin-2-one) – add after autoclave

Fill to 50 mL with deH₂O

Dissolution of PVP will require 2-3 hrs, at 65°C with stirring. Before use allow one hour of stirring and heat to resuspend all salts in the solution.

Sodium acetate solution (3mM)

For 100 samples (10 mL solution)

20.4 mg Sodium Acetate trihydrate (mw = 136.08 g/M)

to 50 mL deH₂O

Auto clave on 'Liquid' setting for 15 minutes.

Phenol-chloroform Isoamyl alcohol (25:24:1) Saturated with 10 mM Tris pH 8.0, EDTA

For 100 samples (50 mL solution) (no need to make, is bought)

25 mL Phenol

24 mL Chloroform (Trichloromethane)

1 mL Isoamyl alcohol

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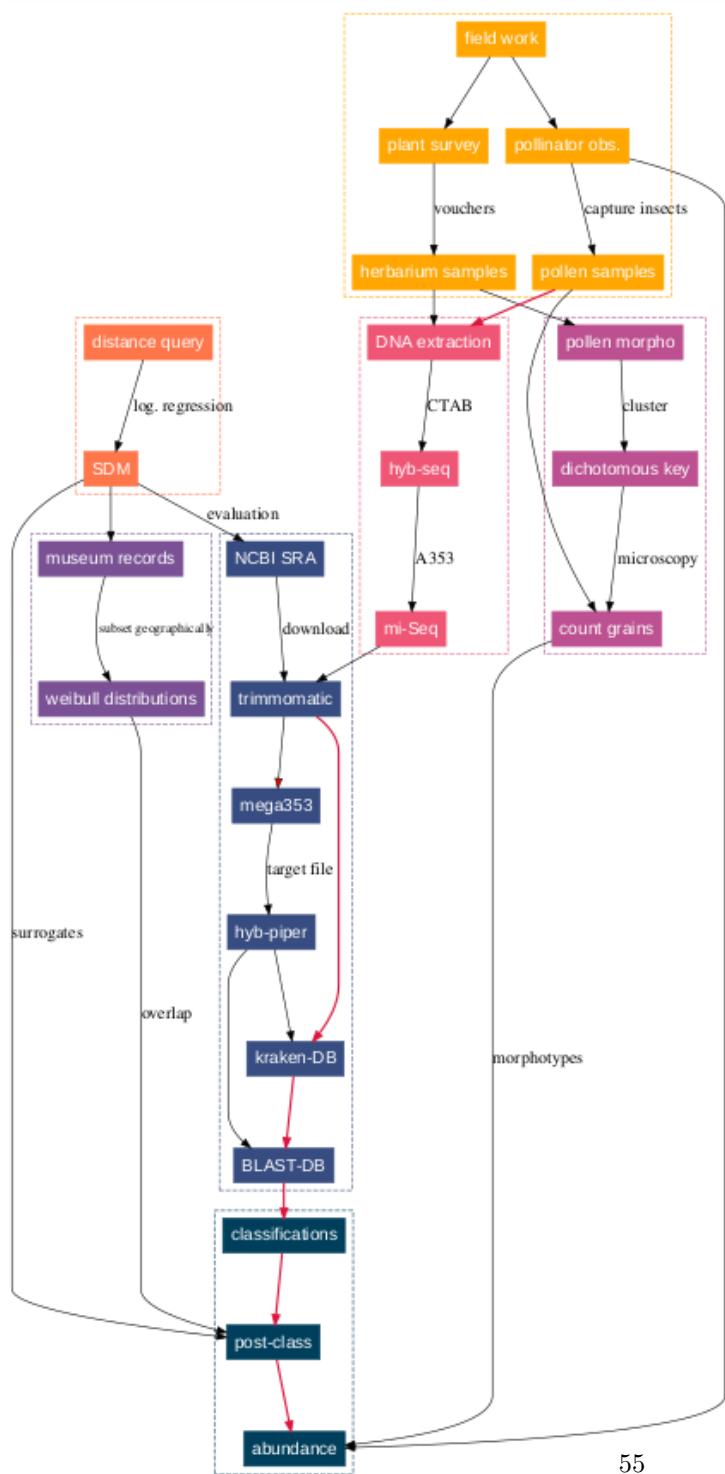
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₇₂₄ THIS SHOULD BE TURNED INTO A SMALLER PNG, AND HAVE THE NUMBER OF SEQUENCED
₇₂₅ METAGENOMIC SAMPLES PLACED INTO THAT COLUMN AND INCLUDED IN TEXT ~~~ NEED
₇₂₆ THIS !!!!

Table 1: Queen Bee Pollen Loads examined

Subgenus	Species	Author	Tongue Length	Microscope Slides	Metagenome Samples
Subterraneobombus Vogt	<i>B. appositus</i>	Cresson 1879	Long	11	NA
Pyrobombus Dalla Torre	<i>B. bifarius</i>	Cresson 1879	Short	11	NA
Thoracobombus Dalla Torre	<i>B. californicus</i>	Smith 1854	Long	8	NA
Pyrobombus Dalla Torre	<i>B. flavifrons</i>	Cresson 1864	Medium	13	NA
Pyrobombus Dalla Torre	<i>B. mixtus</i>	Cresson 1879	Short	3	NA
Bombias Robertson	<i>B. nevadensis</i>	Cresson 1874	Long	5	NA
Cullumanobombus Vogt	<i>B. rufocinctus</i>	Cresson 1864	Short	13	NA
Pyrobombus Dalla Torre	<i>B. sylvicola</i>	Kirby 1837	Short	1	NA

^a All subgenera follow the system of Williams et al. 2008, and placements were found from the NMH website.^b Tongue Lengths collected from Pyke et al. 2012



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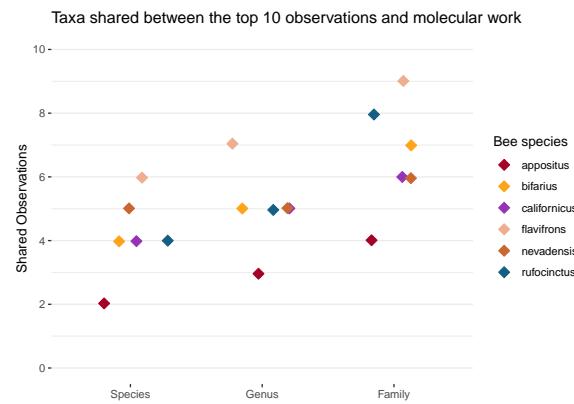
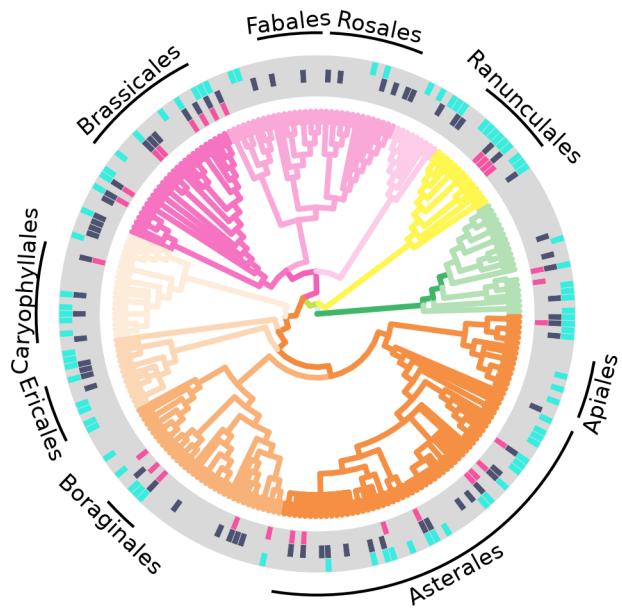


Figure 1: Number of the ten most commonly visited plants which are also in the top ten most common sequences

Biotically pollinated plant genera with morphological or molecular data



Status lacking observed sequenced slide

Figure 2: Phylogenetic tree of all biotically pollinated plant genera in the study area. The innermost ring indicates every genus which Queen Bee's were observed to visit. The intermediate ring indicates that at least a single morphological pollen voucher slide was prepared for a member of the genus. The outermost ring indicates that sequence data were available for at least a member of that genus. Branch colors follow APG 4.

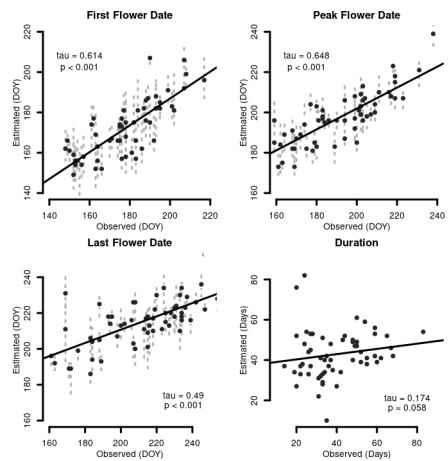


Figure 3: Modelled dates of when major flowering events occurred compared between long term and modelled data

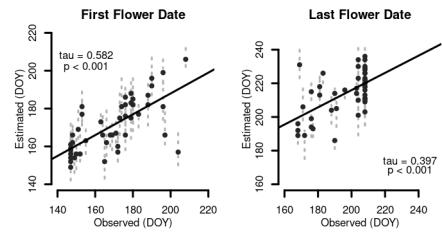


Figure 4: Modelled dates of when major flowering events occurred compared between 2015 and modelled data

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Table 2: Logistic regression assessing accuracy of SDMs

Metric	Value	Metric	Value
Accuracy (Training)	83.75	F-Score	0.84
Accuracy (Test)	84.00	AUC	0.92
Recall	81.03	Concordance	0.92
True Neg. Rate	86.97	Discordance	0.08
Precision	88.04	Tied	0.00

Table 3: Species Distribution Modeling evaluation contingency table

		Training		Testing	
		Absence	Presence	Absence	Presence
Absence	25620	3838	11130	1653	
	6614	28248	2758	12024	

Table 4: Post classification of Sequences via Taxonomy and Ecology, top 15 most abundant reads

Condition	No. Class.	Prcnt. Class.	Total Seqs	Rank
A	143	21.0	32.0	Species
B	205	30.1	10.5	Species
C	5	0.7	0.4	Genus
G	29	4.3	7.8	Species
H	280	41.2	47.9	Genus
None met	18	2.6	1.4	Multiple