

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

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⁴ **Abstract**

5) DNA Barcoding has been remarkably successful in nearly all kingdoms of life and has allowed
6 for the rapid analysis of ecological assemblages. Successful DNA barcoding in the plant kingdom
7 has been more difficult than other kingdoms. Due to this understanding plants in ecological
8 contexts and understandings of their syncology in some instances may begin to lag behind other
9 kingdoms.

10
11 2) Here we utilize hyb-seq, museum studies, and species distribution modelling, to detect the plant
12 species present in pollen loads collected from Queen Bumble Bees.

13
14 3) By utilizing Species distribution modelling we allow for one to process hyb-seq data, create user
15 specified sequence databases which may use MORE ACCURATE alignment algorithms on personal
16 computers over realistic time periods.

17
18 4) We show that hyb-seq using the Angiosperms 353 probes, which are currently being used in the
19 largest ever plant systematic endeavor, offers significant promise to metagenomic approaches in

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20 real world scenarios.

21

22 5) We conclude that these probes offer promise for the identification of plant tissue in both single
23 sample, and metasample contexts.

24 **1 | INTRODUCTION**

25 The inability to reliably identify plants down to species can limit our understanding of ecosystem function
26 and interactions (Bortolus (2008)). This is especially true for genera where species are defined based upon
27 ecological and behavioral rather than morphological properties, and hence can serve as key habitat bioindi-
28 cators (e.g. different species of Sagebrush- *Artemisia* L., Willows - *Salix* L., and Sedges - *Carex* L.) (Gage &
29 Cooper (2013)). The lack of species level data can hinder our understanding of the breadth of habitat which
30 some species occupy, and the interactions they have with other species. Current methods to ameliorate this
31 situation include: ignoring these ecologically relevant levels of detail, revisiting plots as diagnostic mate-
32 rial becomes temporally available, assistance from taxonomic specialists, or the use of barcoding or other
33 molecular techniques.

34 The identification to species is often mired by lack of diagnostic characters (e.g. flowers, fruits, roots or
35 combinations thereof), an increasing lack of taxonomic experts (Hebert *et al.* (2003)) and increasingly the
36 description of cryptic species (Janzen *et al.* (2017), Oliver *et al.* (2009)). And revisiting field sites to identify
37 material using morphological or chemical approaches, can be resource intensive and often does not work.

38 Recently barcoding (the identification of a sample from a single organism *e.g.* a piece of leaf), and metabar-
39 coding (the identification of a sample containing a mix of organisms *e.g.* soil), have shown considerable
40 promise in all Kingdoms of life (Ruppert *et al.* (2019)). With plants the identification of members of cer-
41 tain clades using barcoding has been quite successful (Kress (2017)), whereas with other clades results have
42 been elusive (Liu *et al.* (2014), Group *et al.* (2011), Coissac *et al.* (2012)), however metabarcoding incurs
43 additional challenges to those which exist for the currently implemented barcodes (Li *et al.* (2015), Kress &
44 Erickson (2007), Group *et al.* (2009), Coissac *et al.* (2012)). Particular challenges with barcodes include the
45 utilization of high-copy number sequences are associated with their rates of divergence, gene tree conflict,
46 and hybridization (Coissac *et al.* (2016), Fazekas *et al.* (2009)).

47 Currently the largest plant systematic endeavor ever undertaken, the Kew Plant and Fungal Tree of Life
48 (PAFTOL), is approaching completion (Baker *et al.* (2021a)). This data set will contain hybridization
49 capture (Hyb-Seq) data from at least one species representing each genus in the plant kingdom using the

50 popular A353 probes (Baker *et al.* (2021a)), resulting in over 14,000 represented species. These publicly
51 available data serve to provide a taxonomically comprehensive backbone for plant metabarcoding, and the
52 A353 probes are currently being used in many other plant phylogenetic issues increasing the sampling depth
53 of many clades (Baker *et al.* (2021b)). Data from the 10kP project, which seeks to develop reference
54 genomes from a phylogenetically diverse suite of plants will contribute many more records upon it's intended
55 completion, now slated to be by 2030, similar projects which seek to sequence high amounts of genomes in
56 regions e.g. the 'Darwin Tree of Life' are being undertaken which will contribute data applicable to enormous
57 spatial domains (Cheng *et al.* (2018), Life Project Consortium *et al.* (2022), Lewin *et al.* (2022)). These
58 data will promote the ability to apply metabarcoding to resolve a diversity of questions relevant to theoretical
59 and applied ecology (Kress (2017)). However, the application of metabarcoding still face challenges relating
60 to the enormity of the genomic data sets and the computational power required to process sequence data.
61 Herein we have resolved major components of the problems of identifying plant material without diagnostic
62 morphological character states using the Angiosperms353 (A353) Hyb-Seq probes (Johnson *et al.* (2019)),
63 and custom species sequence databases derived via species distribution modelling, and temporal filtering.

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

78 Considerable amounts of species interactions vary along time (CaraDonna *et al.* (2021)). For the tropics
79 and subtropics, contrasts in the flowering periods of many plant species, can provide an additional filter for
80 identifying material in many metagenomic samples (Janzen (1967), Newstrom *et al.* (1994)). In temperate
81 regions, pollination interactions also vary temporally (CaraDonna *et al.* (2017)), however the overall shorter

82 extent of the active growing season in these systems results in the presence of few to any natural breaks
83 in these systems which reduces the utility of these to operate as filters in the post-processing of sequence
84 matches. Nonetheless, we work through a process which seems applicable to the tropics and subtropics to
85 utilize the temporal dimension for classifying sequencing results.

86 To test these metagenomic and informatics approaches to determine whether the foraging record of Queen
87 Bumble Bee's is consistent across direct observations and the pollen record, an incongruency in several floral
88 visitation networks involving smaller bodied fauna (Barker & Arceo-Gomez (2021), Zhao *et al.* (2019),
89 Alarcón (2010)). The assessment of the plant species compositions of pollen is a desired results, with several
90 applications, and numerous complications (Poron *et al.* (2017), Bell *et al.* (2017), Sickel *et al.* (2015),
91 Bell *et al.* (2019), Suchan *et al.* (2019), Johnson *et al.* (2021)). The two foraging phases of the Queen
92 Bumble Bee life cycle is essential to 1) increase their weight before diapause, 2) increase their ovary weights
93 while establishing their recently found nests, both of these time periods represent potential demographic
94 bottlenecks in bumble bee populations (Sarro *et al.* (2022)). Bumblebees are one of the only groups of insects
95 with unequivocal quantitative evidence for numerous populations declines, while simultaneously serving as
96 the most effective pollinators in temperate montane ecosystems (Cameron & Sadd (2020), Goulson *et al.*
97 (2008), Williams (1982), Colla *et al.* (2012), Bergman *et al.* (1996), Bingham & Orthner (1998)). Montane
98 areas often represent the most diverse areas in the temperate and oftentimes offer the sole potential refugia
99 for multiple dimensions of biodiversity under climate change, whilst simultaneously experiencing the greatest
100 proportional changes in mean annual temperature (Brito-Morales *et al.* (2018), Pepin *et al.* (2022)). An
101 immediate understanding of how to manage previously overlooked keystone insect species, such as bumble
102 bees, is essential if the refugial potential of the temperate mountains are to be utilized while maintaining
103 their current diversity (Loarie *et al.* (2009), Dobrowski & Parks (2016))

104 2 | METHODS

105 Study System & Field Work

106 Observations and sample collection was conducted at The Rocky Mountain Biological Laboratory (RMBL;
107 38°57.5" N, 106°59.3" W (WGS 84), 2900 m.a.s.l.), Colorado, USA (APPENDIX 1 for site information). Pol-
108 linator observations of *Bombus Latreille* spp. (Apidae Latreille) were conducted from June - August of 2015
109 in six study sites characterized by high-montane/subalpine Parkland vegetation communities. Observations
110 of *Bombus* foraging took place for one hour at each field site in three 100m transects, where all flowers were

111 also counted and placed into abundance bins. Corbiculae loads were, non-lethally, collected once from all
112 Queen individuals encountered.

113 **2.1 | Spatial Analyses**

114 **2.1.1 Candidate Species** We downloaded from the Botanical Information and Ecology Network ‘BIEN’
115 (Maitner (2022)) all records adjacent to the field sites to develop an ecologically relevant list of vascular
116 plant species, with expected biotic pollination, which may be present at the study area . We then generated
117 Species Distribution Models (SDMs) to predict their distribution throughout the study area. These maps of
118 potential distribution served as a reference to reduce the list of species to include in the genomic sequence
119 databases.

120 In order to minimise the number of species for which SDM’s were to be generated, BIEN was queried at
121 a distance of up to 100km from our study area and all plant species records were downloaded. In order
122 to emulate the stochasticity of botanical collecting, this data set was bootstrap re-sampled 250 times, with
123 90% of samples selected, to create a testing data set. The median of the logistic regression assessing the
124 probability of occurrence of a species record as a function of distance from the study area was used as a
125 threshold distance, under which, to include species as candidates for distribution modelling.

126 **2.1.2 Distribution Modelling** We used all occurrence records from BIEN ($n = 23,919$) within a 50km
127 border of the Omernik level 3 ecoregion, which includes the study area (*No. 21 “Southern Rockies”*) to
128 construct the species distribution model (Omernik (1987)). These records were copied into two, initially
129 identical, sets, one for generating machine learning models (ML; Random Forest, and Boosted Regression
130 Tree’s), and the other for Generalised Linear (GLM) and Generalized Additive Models (GAM) (Barbet-
131 Massin *et al.* (2012)). **Ensembled predictions have been shown to outperform their constituent**
132 **models, on average, and to reduce the ecological signal to the analytical noise of individual runs**
133 **(Araujo & New (2007)). No single method of producing SDMs has been shown to universally**
134 **outperform others when faced with a large and diverse number of applications, in our case a**
135 **great number of species with differing biologies and ecologies (Elith* *et al.* (2006), Qiao *et al.***
136 **(2015)). In the spirit of these findings, multiple families of models, which can be generated**
137 **together as they have similar requirements regarding the number and ratios of Presence to**
138 **Absence records were ensembled together (Barbet-Massin *et al.* (2012)).**

139 We then generated 4,029 absence points , locations where the focal taxon is anticipated missing, through a
140 random stratification of 19% of the land cover in the area and included them in (BLM CITATION - need

appropriate format for journal). To achieve a larger absence data set, we generated 1,000 pseudo-absence records for each taxon by randomly selecting coordinates located at least 10km away from an occurrence record. For ML models, these pseudo-absences were reduced so that the ratio of presence to absence records were balanced (Barbet-Massin *et al.* (2012)). To achieve this, we removed absence records inside of 10% of the mean sample value of the presence records; the required number of absence records were then randomly sampled.

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

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

535 species were modelled using Generalized Linear Models and Generalized Additive Models. 534 species were modelled using Random Forest and Boosted Regression Trees. To evaluate the accuracy of the species distribution models, additional presence records from GBIF (n = 61,789), and AIM (n = 12,730) were used as test and training sets (n = 74,519) for logistic regression (Ocdownload Gbif.Org (2021), Land Management (2019)). Additional novel absence records were generated from the AIM data set to create a data set where each species has balanced presence and absences. 11 or more paired presence and absence records were required for this testing, resulting in 334 species being included in the logistic regression (Mdn = 110.0, \bar{x} = 223.1, max = 1568 record pairs used) with a 70% test split (Kuhn (2022)).

¹⁷⁰ **2.2 | Molecular Lab Work**

¹⁷¹ All lab work was carried out at The Daniel F. and Ada L. Rice Plant Conservation Science Center at the
¹⁷² Chicago Botanic Garden, Glencoe, Illinois, U.S.A.

¹⁷³ **2.2.1 | Reference Plant Library Generation** Using five years of observational data on *Bombus* Queen
¹⁷⁴ Bee foraging at these studies sites, we identified the plant taxa most frequently visited by Queens across
¹⁷⁵ all years. We sequenced the 12 most commonly visited taxa twice using samples from one site within the
¹⁷⁶ Gunnison River Drainage and one individual from another population. In addition, for any of these 12 focal
¹⁷⁷ species which did not have a congener pair in this filtered sample, we included a congener - or a species from a
¹⁷⁸ closely related genus to serve as an outgroup. We also sequenced another 15 abundant taxa commonly visited
¹⁷⁹ by *Bombus* workers, based on the aforementioned data set (*APPENDIX 4*). Plant collections were identified
¹⁸⁰ via a variety, and typically a combination, of dichotomous keys and primary literature as required (Flora of
¹⁸¹ North America Editorial Committee (1993+), Hitchcock & Cronquist (2018), Ackerfield (2015), Lesica *et al.*
¹⁸² (2012), Cronquist *et al.* (1977+), Allred & Ivey (2012), *Jepson flora project* (2020), Mohlenbrock (2002)).

¹⁸³ **2.2.2 | Plant Genomic DNA Extraction** Plant genomic DNA was isolated from ~ 1 cm² of leaf tissue
¹⁸⁴ from silica-gel dried or herbarium material using a modified cetyltrimethylammonium (CTAB) protocol
¹⁸⁵ (Doyle & Doyle (1987)) that included two chloroform washes. DNA was quantified using a Nanodrop 2000
¹⁸⁶ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Qubit fluorometer (Thermo Fisher Scientific).

¹⁸⁷ **2.2.3 | Pollen Genomic DNA Extraction** Pollen genomic DNA was extracted from corbiculae using a
¹⁸⁸ CTAB based protocol modified from Lahlamgiah et al. and Guertler et al. (2014, 2014). A SDS extraction
¹⁸⁹ buffer (350 µL , 100mM Tris-HCl, 50 mM EDTA, 50 mM NaCl, 10% SDS v/v., pH 7.5) was added followed by
¹⁹⁰ vortexing to allow dissolution of corbiculae. Pollen grains were then macerated with Kontes Pellet Pestles,
¹⁹¹ and the tip of these washed with 130 µL of the SDS extraction buffer, samples were then incubated for
¹⁹² 1 hour at 30°C. This was followed by the addition of 10% CTAB solution (450ul, of 20 mM Tris-Cl pH.
¹⁹³ 8.0, 1.4 M NaCl, 10 mM EDTA pH 7.5, 10% CTAB, 5% PVP, ~85% Deionized water) and RNAse (10
¹⁹⁴ uL of 10 mg/mL) and samples were incubated for 40 minutes at 37°C, on heat block (Multi-Blok, Thermo
¹⁹⁵ Fisher Scientific, Waltham Massachusetts) set to 40°C. After 20 minutes incubation, Proteinase K (15 µL of
¹⁹⁶ 20mg/ml) and DTT (12.5 µL of 1M in water) were added, and the samples were further incubated at 60°C
¹⁹⁷ for 1 hour. Samples were then incubated overnight at 40°C. 500 µL of Phenol-Chloroform-Isoamyl alcohol
¹⁹⁸ (25:24:1) were added, vortexed, and centrifuged at 10,000 rpm for 10 minutes and the aqueous phase was

199 pipetted to a 1.5 ml centrifuge tube.

200 To precipitate the DNA, chilled Isopropyl alcohol & 3 mM Sodium acetate (5:1) equivalent to 2/3 of the
201 volume of sample were added, with 1 hour of chilling at -20°C, followed by 10 minutes of centrifuging at
202 13,000 rpm. The supernatant was pipetted to a new 1.5 ml centrifuge tube, and 70% EtOH (400 µL) were
203 added before chilling at -20°C for 20 minutes followed by centrifugation at 13,000 rpm for 10 minutes. Both
204 tubes were then washed with 75% EtOH (400 µL), inverted, centrifuged at 13,000 rpm for 4 minutes, and
205 the solution discarded, then washed with 95% EtOH (400 µL), inverted, centrifuged at 13,000 rpm for 4
206 minutes, and the solution discarded. Pellets were dried at room temperature overnight before resuspension
207 in Nuclease free H₂O. Extractions were assessed using a Nanodrop 2000 (Thermo Fisher Scientific) and
208 Qubit fluorometer (Thermo Fisher Scientific). DNA extracts were then cleaned using 2:1 v./v. Sera-Mag
209 beads (Cytiva, Little Chalfont, UK) to solute following the manufacturer's protocol, eluted in 0.5x TE, and
210 the eluent allowed to reduce by half volume in ambient conditions. DNA was quantified using a Qubit
211 fluorometer.

212 **2.2.4 | Fragmentation, Library Preparation & Target Enrichment** Library preparation was per-
213 formed using the NEBNext Ultra II FS-DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich,
214 Massachusetts, USA) using slightly modified manufacturers recommendation. Fragmentation was performed
215 at ½ volume of reagents and ¼ enzyme mix for 40 minutes at 37°C, with an input of 500 ng cleaned DNA.
216 Adapter Ligation and PCR enrichment were performed with ½ volumes, while cleanup of products was
217 performed with ½ volume of SPRI beads (Beckman Coulter, Indianapolis, Indiana, USA) and recommended
218 volumes of 80% v./v. ethanol washes. The exception was the herbarium specimens which were not frag-
219 mented and only end repaired, with similar library preparation of all samples. Products were analysed on
220 4% agarose gels, and a Qubit fluorometer. Libraries were pooled and enriched with the Angiosperms 353
221 probe kit V.4 (Arbor Biosciences myBaits Target Sequence Capture Kit) by following the manufacturer's
222 protocol and Brewer et al. 2019. Sequencing was performed using an Illumina mi-Seq with 150-bp end reads,
223 (NUSeq Core, Chicago, Illinois).

224 **2.2.5 | Computational Processes and Analyses.**

225 **2.2.5.1 | Reference Library Data Processing** Sequences were processed using Trimmomatic, which
226 removed sequence adapters, clipped the first 3 bp, discarding reads less than 36 bp, and removing reads
227 if their average PHRED score dropped beneath 20 over a window of 5 bp (Bolger & Giorgi (2014), Tange
228 (2021)). Contigs were generated using HybPiper using target files created by M353 (Johnson *et al.* (2016),

²²⁹ McLay *et al.* (2021)).

²³⁰ **2.2.5.2 | Sequence Identification** A custom Kraken2 database was created by downloading representative species of each genus indicated as being present in the study area by the spatial analyses from the ²³¹ Sequence Read Archive (SRA) NCBI (Wood *et al.* (2019)). These sequences were processed in the same ²³² manner as our novel sequences . The Kraken2 database was built using default parameters. Kraken2 was ²³³ run on sequences using default parameters (*APPENDIX 5*). Following Kraken2, Bracken was used to clas- ²³⁴ sify sequences to terminal taxa (Lu *et al.* (2017)). Results from both Kraken2 and Bracken, results were ²³⁵ reclassified manually to identify terminal taxa. For example, when only a single species of a genus was known ²³⁶ in the study area, but our database used a representative of another taxon in the genus, this species was ²³⁷ coded as the result. The re-coding of sequences from another representative species for the genus to the sole ²³⁸ RMBL representative allowed the identification of XX & % more species. ²³⁹

²⁴⁰ **2.2.5.3 | Identification of Sequence Matching Loci** A local NCBI database was built using the same ²⁴¹ processed novel and downloaded sequences as the previous databases (Camacho *et al.* (2009)).

²⁴² **2.2.5.4 | Morphological Pollen identification**

²⁴³ To develop a reference library of pollen grains which may be present in corbiculae loads, an image reference ²⁴⁴ collection of fuchsin-jelly stained (Beattie (1971)) slides was assembled from slides previously prepared by the ²⁴⁵ authors (n = 21), and other researchers (n = 38) (Brosi & Briggs (2013)). Using five years of observational ²⁴⁶ data on *Bombus* Queen Bee foraging at these studies sites (Ogilvie unpublished), as well as the Vascular ²⁴⁷ Plant Checklist (Frase & Buck (2007)), an additional 62 voucher slides for species were prepared and imaged ²⁴⁸ at 400x (Leica DMLB, Leica MC170 HD Camera, Leica Application Suite V. 4.13.0) from non accessioned ²⁴⁹ herbarium collections to supplement the number of species and clades covered (Appendix 3). ²⁵⁰ We used Divisive Hierarchical Clustering techniques to determine which plant taxa were distinguishable via ²⁵¹ light microscopy, and to develop a dichotomous key to pollen morphotypes. Ten readily discernible categorical ²⁵² traits were collected from each specimen in the image collection. These traits were transformed using Gower ²⁵³ distances, and clustered using Divisive Hierarchical clustering techniques (Maechler *et al.* (2022)). Using ²⁵⁴ the cluster dendrogram, elbow plot, and heatmaps (Hennig (2020)), of these results morphological groups ²⁵⁵ of pollen which could not be resolved via microscopy were delineated, and a dichotomous key was prepared ²⁵⁶ (*APPENDIX NO.*). This key was then used to identify the pollen grains sampled from corbiculae loads to ²⁵⁷ morphotypes in a consistent manner. To prepare the pollen slides from corbiculae, all corbiculae loads were

258 broken apart and rolled using dissection needlepoints to increase heterogeneity of samples. *Cerca* 0.5mm²
259 of pollen was placed onto a ~4mm² fuchsin jelly cube (Beattie (1971)) atop a graticulated microscope
260 slide, with 20 transects and 20 rows (400 quadrants) (EMS, Hartfield, PA). The jelly was melted, with
261 stirring, until pollen grains were homogeneously spread across the microscope slide. Slides were sealed with
262 Canada Balsam (Rublev Colours, Willits, CA) followed by sealing with nail polish; all samples are noted in
263 APPENDIX 3. To identify the pollen present in corbiculae loads, light microscopy at 400x (Zeiss Axioscope
264 A1) was used. In initial sampling in three transects, each pollen grain was identified to morphotype and
265 counted; an additional two transects were scanned for morphotypes unique to that slide, if either transect
266 contained an unique morphotype than all grains in that transect were also identified and counted. Subsequent
267 to the first round osf sampling, non-parametric species richness rarefaction curves (Oksanen *et al.* (2022)),
268 and non-parametric species diversity rarefaction curves were used to assess the completeness of sampling
269 (Chao *et al.* (2014), Hsieh *et al.* (2020)). Slides not approaching the asymptote of the rarefaction curve
270 were then re-sampled, and analysed iteratively for up to a total of seven transects APPENDIX 2.

271 2.3 | Temporal Analyses

272 To estimate the duration of dates in which plant species were flowering weibull estimates of several pheno-
273 logical parameters all spatially modelled taxa were developed (Belitz *et al.* (2020), Pearse *et al.* (2017)).
274 Only BIEN records which occurred in the Omernik Level 4 Ecoregions within 15km of the study area ($n = 5$
275 Level 4 Ecoregions, or conditionally 6 if enough records not be found in the nearest 5), and which were from
276 herbarium records were included. To remove temporally irrelevant herbarium records, i.e. material collected
277 during times which flowering is impossible at the study area due to snow cover, we used the SnowUS data
278 set (Iler *et al.* (2021), Tran *et al.* (2019)) from 2000-2017 was analyzed for the first three days of contiguous
279 snow absence, and the first three days of contiguous snow cover in Fall. Herbarium records after the 3rd
280 quantile for melt, and the 1st quantile for snow cover of these metrics were removed. Species with > 10
281 records had their weibull distributions generated for the date when 10% of individuals had begun flowering,
282 when 50% were flowering, and when 90% of individuals had flowered.

283 **2.4 | Floral Observations**

284 **3 | RESULTS**

285 **3.1 | Spatial Analyses**

286 [Table 1 about here.]

287 [Table 2 about here.]

288 The median (25.009 km) of the logistic regression assessing the probability of occurrence of a species record as
289 a function of distance from the study area was used as a threshold distance to include species for distribution
290 modelling. A 2-sample test for equality of proportions with continuity correction (χ^2 -squared = 13.254, df
291 = 1, p-value = 0.000136, 95% CI 0.04-1.00) was used to test whether more of the records located in the
292 broad ecological sites present at the field station, between the distance of the median (25.009 km) to the
293 third quantile (ca 43.830 km) of the regression distance, where true presences at the field station. Including
294 these records would have resulted in modelling an additional 222 species distributions of which 30 are true
295 presences these taxa were not modelled.

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

298 At the field site, of the 554 vascular plants with biotic pollination syndromes, the 493 ML ensembles accu-
299 rately predicted the presence of 362 (65.3%), incorrectly predicted the presence of 64 (11.6%), incorrectly
300 predicted 34 true presences (6.1%) as being absent, and correctly predicted the true absence of 33 (6.0%).
301 The balanced accuracy of the ensembled models is 0.627 (Sensitivity = 0.340, Specificity 0.914). Of the 554
302 vascular plants with biotic pollination syndromes, the 475 LM ensembles accurately predicted the presence
303 of 286 (51.6%), incorrectly predicted the presence of 41 (14.3%), incorrectly predicted 93 true presences
304 (16.8%) as being absent, and correctly predicted the true absence of 55 (9.9%). The balanced accuracy of
305 the ensembled models is 0.664 (Sensitivity = 0.573, Specificity 0.754). Of the 554 vascular plants with biotic
306 pollination syndromes in the flora 13 (2.3%) were in the Orchid family and 41 (7.4%) are non-natives, both
307 of which are restricted from the database, and can only reduce the number of true predicted presences by
308 roughly 10%.

309 At the six study plots, of the 117 plant species identified to the species level across the spatial extents of all
310 plots and duration of queen bee activity, the ML ensembles predicted the presence of 105 (89.7%) of them,

311 and LM ensembles 102 (87.2%). Of the missing species two (1.7%) are Orchids, six (5.1%) are non-native,
312 and one (0.85%) is of contested taxonomic standing, all of which (7.65%) are restricted from the initial query
313 database.

314 3.2 | Microscopic Pollen identification

315 Using the fuchsin jelly preparation and light microscopic analyses of grains and scoring of 10 character
316 states resulted in the establishment of 28 morphotypes which grains could be classified into. APPENDIX
317 7. 60 samples were counted and based on rarefaction **had over % of expected morphotypes found**
318 (morphotype richness, $\bar{x} = 4.5$, Mdn = 4, min = 1, max = 9), all samples had expected morphotype diversity
319 reach the asymptote APPENDIX 8. The number of counted pollen grains in each sample range from (MIN
320 - 16,293, $\bar{x} = 2788.685$, Mdn = 1453).

321 [Figure 1 about here.]

322 3.3 | Metabarcoding Pollen identification

323 54 corbiculae loads had DNA extracted and underwent various steps towards hyb-seq, in the end a total of
324 44 corbiculae samples were sequenced, 7,752,353 reads were recovered from sequencing. The number of reads
325 per sequence varied widely (range = 76 - 508,795, $\bar{x} = 176,189.8$, Mdn = 138,395). Of the possible 353 loci,
326 the number which were recovered from each sample, and informative to BLAST were range = 24 - 353, $\bar{x} =$
327 305.5, Mdn = 331. The number of reads per loci from across all samples had a range of 178 - 506,653, $\bar{x} =$
328 20,688, Mdn = 12,616.

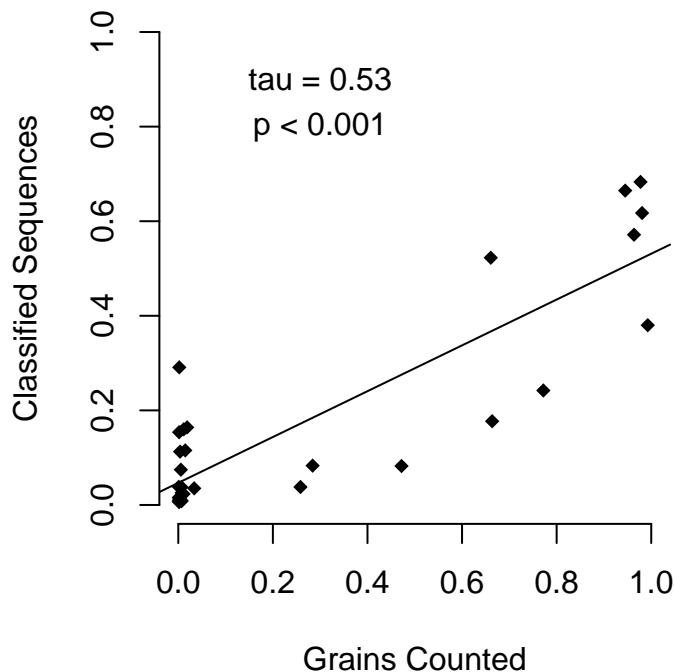
329 APPENDIX X Reads Per Loci.

330 After trimming 7,865,680 sequences remained. 10,682,538 reads were matched using Kraken, of the reads
331 classified by Kraken 10,160,768 reads were matched using Bracken, of the reads classified by Kraken 7,302,876
332 reads were matched using BLAST. Based upon subjective review of the three classifiers **APPENDIX X**
333 **MOLECULAR NETWORKS - 3 DIFFERENT ONES**, BLAST was chosen as the classification
334 method which yielded the most probable results, and it's values were used for all subsequent analyses.

335 To determine at which level species in pollen loads could be detected the results of light microscopy were
336 compared to the molecular results. The pollen samples contained three morphotypes which could readily be
337 identified via microscopy. Two of these mapped to the clades (Boraginaceae & Heliantheae Alliance), and
338 one to a Asteraceae Berchtold & J. Presl less Heliantheae. Boraginaceae grains were detected in 85.7% of

339 samples where the proportion of target grains were between 0.01-1 ($n = 14$ Mdn = 0.572). Asteraceae type
340 1, non-helianthoids, were detected in 50% of samples where the proportion of target grains were between
341 0.001-0.01 ($n = 6$ Mdn = 0.002) Asteraceae type 2, Helianthoids, were detected in 62.5% of samples where the
342 proportion of target grains were between 0.001-0.01 ($n = 8$ Mdn = 0.003). Both morphotypes of Asteraceae
343 pollen were detected in 100% of samples where the proportion of target grains were between 0.01-1 ($n = 3$
344 Mdn = 0.011), and Ericaceae were detected in 50% of samples where the proportion of target grains were
345 between 0.001-0.1 ($n = 2$ Mdn = 0.01).

Correlation of Proportion Counted Grains and Sequence Reads



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

352 The relationship between the number of pollen grains in a sample and the number of sequence reads is roughly
353 *curvilinear*, where grains which are present in trace amounts are overestimated by sequence counts, while
354 grains present in high amounts are underestimated. This is likely due to the proportion of high false positives
355 which occur in the classification process with NGS (BELL NOVEMBER 2021). There was strong evidence

357 of a strong correlation between the proportion of grains per morphotype and the number of sequences per
358 group (0.53, $p < 0.0001$, $n = 31$).

359 3.4 | Temporal Analyses

360 The first date of modeled snow melt in the Gothic area ($n = 17$, $\bar{x} = 137.9$, $Mdn = 135$, 3rd quantile = 151),
361 and the first date of a consistent winter snow base ($n = 17$, $\bar{x} = 299.9$, $Mdn = 300$, 1st quantile = 291) from
362 2000-2017, were used as delimiters for the inclusions of herbarium records in modelling. Of the **500** species
363 predicted likely present in the area via logistic regression, 332 species (64.4%) with more than 10 records in the
364 focal level 4 ecoregions ($\bar{x} = 35.01657$, $Mdn = 35$, max = 96) had weibull estimates calculated, an additional
365 56 species (11.2%) with enough contributing records from the “Sedimentary Mid-Elevation Forests”, a large
366 ecoregion in general just beneath the elevation bands occupied by the five ecoregions around the study area
367 had weibull estimates also calculated ($\bar{x} = 13.86885$, $Mdn = 13$, max = 24).

368 Only 58 of these 388 species ($n = 34.56897$, $Mdn = 31$) were able to be compared to plot based observational
369 data from the long term (1974–2012) data set. Of these species relatively high accord was observed between
370 the long-term ground truthed data set, and the modelled species. There was very strong evidence that the
371 weibull estimates were positively associated with the observed onset ($r^2 = 0.72$, $p < 0.0001$, $\tau = 0.61$)
372 and peak ($r^2 = 0.70$, $p < 0.0001$, $\tau = 0.65$) of flowering, and that the number of herbarium samples had
373 a moderate effect on the estimates ($p = 0.004$ and $p = 0.034$ respectively). There was very strong evidence
374 that the weibull estimates had a positive association with the observed cessation of flowering ($r^2 = 0.4339$, p
375 < 0.0001 , $\tau = 0.489$), however their was no evidence that sample size had an effect ($p = 0.349$). There was
376 moderate evidence that the weibull estimates, with an effect of sample size, had a weak positive association
377 with the observed duration of flowering ($p = 0.0401$, $r^2 = 0.07$, $\tau = 0.17$).

378 [Figure 2 about here.]

379 3.5 | Floral Observations

380 The six sites were surveyed for a total of 52 hours from May 27-July 27. A total of 723 queen-pollen foraging
381 interactions were observed (range per bee species by week range = 1 - 18, $\bar{x} = 3.46$, $Mdn = 2$), with a
382 range of total observed interactions per bee species across this time period (min = 1, $\bar{x} = 59.08$, $Mdn = 19$,
383 max = 184). Plants varied widely in the number of interactions which they partook in with each species
384 of bee (range per plant species by week min = 1 - 20, $\bar{x} = 3.51$, $Mdn = 2$), with a range of total observed

385 interactions per plant species over this time period (min = 1, $\bar{x} = 20.26$, Mdn = 4, max = 141). The number
386 of plant species which bees were observed interacting with varied more narrowly (range = 1 - 18, $\bar{x} = 8$,
387 Mdn = 6).

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

389 [Figure 3 about here.]

390 3.6 | Integrated Observational, Molecular, and Palynological Network

391 For example a common UNKNOWN sequence mapped to the Asteraceae family, but which was flagged
392 by temporal filters and is present in both *B. nevadensis* Cresson and *B. rufocinctus* Cresson pollen is
393 most likely *Frasera* Walter, failed extraction. A similar likely mismatch could be between what was fide
394 molecular evidence as *Agastache pallidiflora* (A. Heller) Rydb. but where feeding was infrequently observed
395 on *Pedicularis* L., likely due to this entire order being represented by only a single molecular reference species.

396 It is not unlikely that much of the difference in the results between the observational and molecular work
397 are attributable to the challenges in detecting rare events in these smaller sizes. For example, no more than
398 10 bee corbiculae loads per species were sequenced with the Mdn = 7 . . . , and the median of interactions
399 with the top 5 plant sizes constituted 0.8142857 of the top.

400 ... many of our results indicate foraging on *Viola* L. spp, zygomorphic flowers with architecture which
401 would require subtle handling and strength to reach the pollen and nectar loads. . . . Or the *Epilobium* L.
402 spp. results indicating that a species of *Chamerion* Seg. such as *C. angustifolium* (L.) Scop. or *latifolium*
403 (L.) Sweet is occasionally utilized, as it supported by limited paylnological data.

404 4 | DISCUSSION

405 We have demonstrated how Angiosperms533 hyb-seq probes may be used for plant barcoding in a metage-
406 nomic context. This was exemplified in an ecologically relevant scenario, where the results have immediate
407 implications for natural history driven fundamental science and the applied science of land management. The
408 test pollen loads contained a number of closely related taxa, some in notoriously morphologically difficult
409 clades with rapid rates of diversification (e.g. *Mertensia*, *Lupinus* L.), at naturally occurring proportions
410 (Nevado *et al.* (2016), Nazaire & Hufford (2014)). We incorporated spatial and temporal approaches for cre-
411 ating custom sequence databases an approach which is readily applicable to any lab group with the capacity

412 to perform next-generation sequencing across the entirety of multiple continents, and which we expect to be
413 highly beneficial in many study areas. By combining insights from these novel approaches with an extensive
414 observational field based study we show how these methods may be applied to test a variety of hypotheses
415 related to ecological interactions.

416 We anticipate that many of the complications which we faced, using opportunistically collected pollen loads
417 and the first implementation of this method may readily be overcome. It seems apparent that we had
418 issues detecting pollen from several genera of plants, based upon these and other observational studies most
419 likely *Vicia* L., *Lathyrus* L., and *Frasera* (Inouye (1980), Pleasants (1980)), this is most likely related to
420 user error in obtaining high quality DNA during the plant reference library generation period. (**REED**
421 **SHOULD HUNT FOR LOCI RETURNS FROM BLAST LIKE HE DID POLLEN**). Additional
422 complications seem to relate to the presence of closely related false positives, e.g. frequent classifications
423 of sequences as *Trollius* L., *Caltha* L., and *Thalictrum* L. alongside a more common species in the family,
424 e.g. *Delphinium*. L. Many of our errors are known to us and multiple mnemonics are in *APPENDIX XX*
425 to assist others in future attempts to achieve better results. However, the line between false and negative
426 positives may be blurred in some of these instances and warrant further work, for example **Ericaceae pollen**
427 **grains were observed in a number of samples in trace quantities....**

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

431 We have concerns regarding the number of persons training to become and practice botany, and grave
432 concerns regarding the funding mechanisms for floristic and field based botanical research and for centralized
433 authorities to produce consensus opinions on alpha taxonomy (Prather *et al.* (2004b), Kramer & Havens
434 (2015), Prather *et al.* (2004a), Crisci *et al.* (2020), Manzano (2021), Stroud *et al.* (2022)). To reduce
435 the effects of a low population density of botanists on the maintenance of and production of flora's and
436 to foster meta-genomics across landscapes without field stations we utilized Species Distribution Modelling
437 to generate predictive species lists. In this proof of concept example we performed several iterations of
438 modelling runs, and several approaches (i.e. the 'linear models', and the 'machine learning'), which took
439 notable amounts of compute power. We suspect the possible deleterious nature of this endeavor may be
440 reduced by: 1) more field surveying by crews will reduce the need to generate as many species 2) fewer runs
441 of models, 3) only running machine learning models which do not require an explicitly process to reduce
442 spatial autocorrelation. However, given the time required to perform all aspects of a study, even our amount
443 of computation was negligible. Further, we are very optimistic about the possibility for persons to perform

these tasks, as mentioned we utilized roughly only one quarter of the records which were digitally available for presence, and we suspect others will have enough records to perform this process nearly anywhere else in the temperate. Tandem to the lack of continued expertise required to generate and maintain species lists, is the expertise required to continue tracking when major phenological events occur in many plant species at relatively fine scales or under novel climates. Knowledge of these events is currently limited to general time periods of only a handful of phenological events and groups of organisms (e.g. flowering initiation, or trees) (Prather *et al.* (2004a), Li *et al.* (2016)). While many programs and initiatives exist to collect phenological information on subsets of easily identifiable charismatic species to detect major trends in phenology, by design these capture only a subset of the extent diversity (Betancourt *et al.* (2005), Havens *et al.* (2007)). In many instances it appears that while landscapes respond similarly to environmental variables which predict phenological responses, that individual species vary widely in their responses to similar environmental cues, or respond to different cues (Augspurger & Zaya (2020), Xie *et al.* (2015), Xie *et al.* (2018), CaraDonna *et al.* (2014)). As can be seen here, predictions of when a single, major phenological event occurs is already data limited, with sample size having an effect on the subset of species which we could even generate weibull estimates for.

The nearly complete Plant and Fungal Tree of Life (PAFTOL) will provide a comprehensive phylogenetic backbone of the entire plant kingdom, and the inclusion of A353 probes with lineage specific probe sets is common in producing massive genetic datasets (Baker *et al.* (2021b)). We predict that the A353 probes which it is utilizing to work nearly immediately for DNA barcoding of whole plant material, and that more elaborate validation studies in controlled metabarcoding settings, utilizing existing experimental designs, will have favorable results (Bell *et al.* (2017), Bell *et al.* (2019), Bell *et al.* (2021), Lamb *et al.* (2019)). In particular the harvesting of loci with more variation in certain lineages, and or with more variable flanking regions, will prove promising for identifying closely related plant material (CITE). We suspect that conserved reaches of genes resulted in the high amounts of reads in somewhat obscure species. Given that the A353 loci are nuclear, single copy, and a variety are present the possibility of identifying target loci for quantitative purposes is high, without continual PCR enrichment is possible; this would align with relatively high efficacy of WGS (Lang *et al.* (2019), Peel *et al.* (2019), Bell *et al.* (2021)). Recent evidence indicates that the potential for identifying nearly cryptic taxa and even infra-specific inference, of either whole plant material, and perhaps in metagenomic context are possible (Ottenlips *et al.* (2021), Wenzell *et al.* (2021), Loke et al. in prep, Slimp *et al.* (2021), Beck *et al.* (2021)).

The remarkably clear foraging preferences of *Bombus*, both here and across a great many localities globally, reiterates the needs for land managers to maintain relatively high amounts of members of the Fabaceae,

476 Boraginaceae, and Ranunculaceae across the landscape. Numerous historic, and some ongoing, land man-
477 agement actions reduce the ability of many landscapes to support stable populations of *Bombus*. Historic,
478 and occasionally current, livestock grazing may be associated with the targeted removal of many species of
479 plants which are known to have compounds toxic to cattle. In particular, the removal of locoweeds (Fabaceae:
480 *Astragalus* L. & *Oxytropis* DC.) and larkspurs (Ranunculaceae: *Delphinium*) were common across public lands
481 administered by the United States Forest Service, and to an extent the Bureau of Land Management (Ralphs
482 & Ueckert (1988), Aldous (1919), Ralphs *et al.* (2003)). Further actions, generally initiated by settlers to
483 gain water rights or to support mining operations, involved the channelization and incising of streams, and
484 culling of beavers, reducing the extent of wetlands and associated mesic meadows which provide habitat for
485 many species of *Mertensia* (Boraginaceae) widely distributed across Western North America (). Fire sup-
486 pression further resulted in the succession of many Aspen groves to Conifer stands, decreasing the mosaic of
487 age structured habitats in many landscapes, adversely effects habitat for tall *Mertensia* species. Finally the
488 effects of Nitrogen deposition, especially given the West's growing population still pose adverse effects on the
489 abundance of a variety of species of Fabaceae (see Stevens *et al.* (2018)). Current solutions to these issues,
490 involve targeted burns, reintroduction of beavers and beaver habitats, and the possibility of re-seeding a
491 variety of 'locoweeds' and 'larkspurs' in areas now seldom used for grazing. The highly enthusiastic response
492 of land managers, and homeowners, to plant *Asclepias* L. to improve Monarch Butterfly (*Danaus plexippus*
493 L.) habitat provides an effective framework for the latter.

494 5 | CONCLUSION

495 We believe that the combination of spatial and temporal models, united and guided by localized natural
496 history knowledge, provides the essential components of a bayesian framework for approaching the coarse
497 elucidation of ecological interactions using DNA Barcoding. Herein we crudely utilized this thinking via
498 binary outcomes, should a species predicted be predicted present or not? Is it unequivocally flowering
499 or not? Myriad data show biological systems and ecological interactions have more variance than can be
500 reasonably discretely parsed. We expect that within a bayesian framework studies of pollinator behavior
501 may be enacted via this approach at a landscape level, e.g. the scale of an entire drainage basin such as the
502 Gunnison which is quickly becoming one of the worlds few model ecosystems. We hope that the promise of
503 A353 probes as tools for metabarcoding play a role in these endeavors.

504 **AUTHOR CONTRIBUTIONS:** R.C.B conducted botanical collections, conducted all molecular lab
505 work, lead all analyses, and writing. J.E.O conceived, designed, and conducted all ecological fieldwork,

506 assisted with analyses, and writing. E.J.W. prepared, imaged, and collected trait data on pollen reference
507 slides, and assisted with analysis of trait data and writing a dichotomous key. S.T. assisted with spatial
508 analyses and writing. P.J.C assisted with ecological analyses and writing. J.B.F. conceived, and designed all
509 lab work, analyses, and integration of approaches, assisted with writing, and secured funding for molecular
510 work.

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

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

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

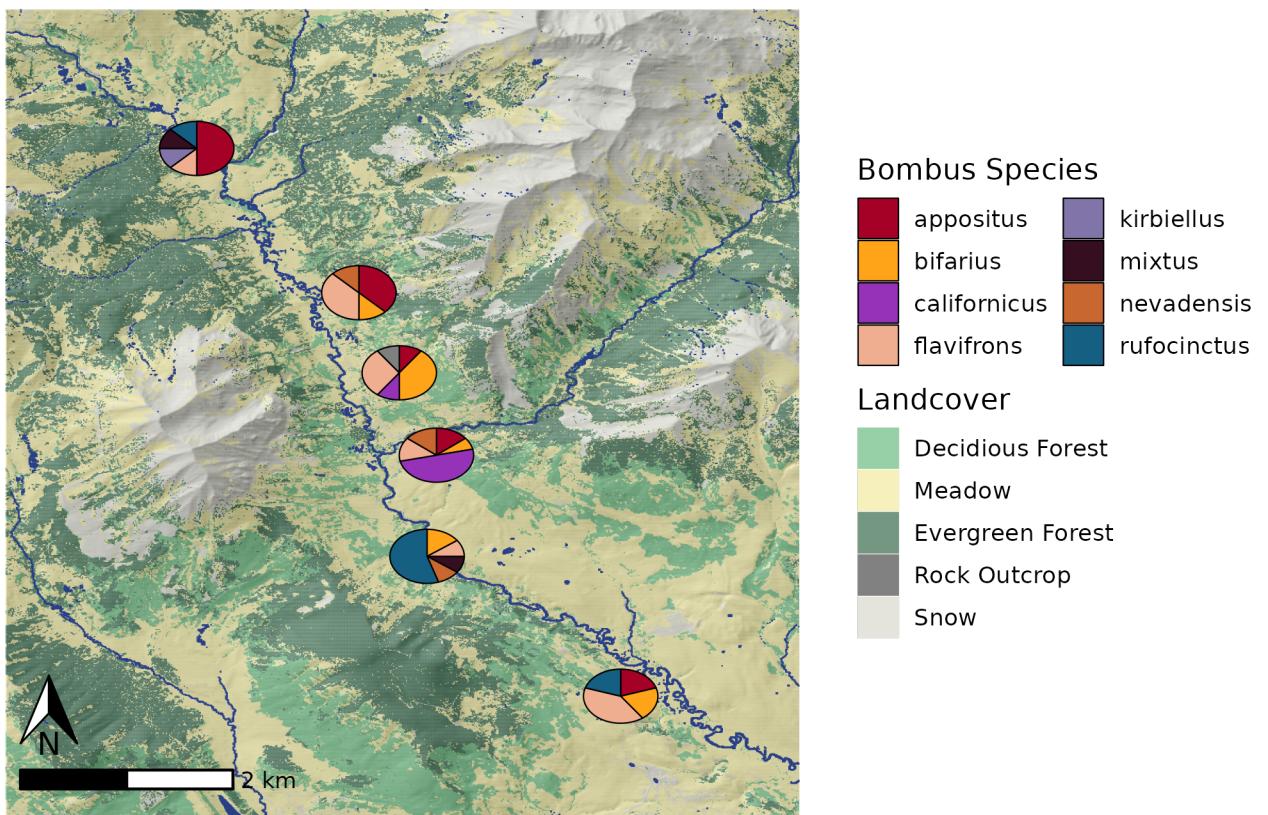
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532 **References**

533 **Supporting**

Origins of Corbiculae Loads



Upper East River Valley, Colorado

536 Appendix 2 - Species Distribution Models Predictors

Layer	LM	Description	Source
1.		Mean annual cloudiness - MODIS	Wilson et al. 2016
2.		Cloudiness seasonality 1 - MODIS	Wilson et al. 2016
3.		Cloudiness seasonality 2 - MODIS	Wilson et al. 2016
4.		Cloudiness seasonality 3 - MODIS	Wilson et al. 2016
5.		Beginning of the frost-free period	Wang et al.
6.		Climatic moisture deficit	Wang et al.
7.		Degree-days above 5C from	Wang et al.
8.		Mean annual precipitation	Wang et al.
9.		Mean annual precipitation as snow	Wang et al.
10.		Temperature seasonality	Wang et al.
11.		2015 Percent Grass/Herbaceous cover - MODIS	(MOD44B)
12.		2015 Percent Tree cover from Landsat 7/8	(GLCF)
13.		Soil probability of bedrock (R Horizon)	SoilGrids
14.		Soil organic carbon (Tonnes / ha)	SoilGrids
15.		Surface soil pH in H ₂ O	SoilGrids
16.		Surface soil percent sand	SoilGrids
17.		Soil USDA class	SoilGrids
18.		Topographic elevation	EarthEnv DEM
19.		Topographic elevation, moving window.	EarthEnv DEM
20.		Topographic percent slope	EarthEnv DEM
21.		Topographic wetness index	EarthEnv DEM
22.		Topographic aspect from	EarthEnv DEM
23.		Annual potential solar radiation computed	r.sun
24.		Estimated actual (w-/cloud) solar radiation r.	sun / Wilson et al. 2016
25.		Log-transformed distance to surface water Gl	Global Surface Water Explorer
26.		Percent surface water Gl	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>Helianthus 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>Mertensia ciliata</i> (James ex Torr.) G. Don	Boraginaceae	ID 1754185	S	Idaho, Valley	18.VI.2018	tba	979.3
<i>Mertensia 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>Mertensia fusiformis</i> Greene	Boraginaceae	RMH 720600	P	Colorado, Gunnison	9.VII.1997	tba	38.9
<i>Campanula rotundifolia</i> L.	Campanulaceae	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>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>Lupinus bakeri</i> Greene	Fabaceae	CHIC tba	S	Colorado, Gunnison	18.VII.2020	tba	3.6
<i>Vicia americana</i> Muhl. ex Willd.	Fabaceae	CHIC tba	S	Montana, Carbon	4.VII.2019	tba	10020.8
<i>Vicia americana</i> Muhl. ex Willd. var. minor Hook.	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>Frasera speciosa</i> Douglas ex Griseb	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 capitatum</i> Douglas ex. Benth	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>Hydrophyllum fendleri</i> (Gray) Heller	Hydrophyllaceae	CHIC tba	S	Arizona, Coconino	17.VII.2020	tba	617.7
<i>Agastache pallidiflora</i> (Heller) Rydberg	Lamiaceae	CHIC tba	S	Arizona, Coconino	17.VII.2020	tba	617.7
<i>Chamerion angustifolium</i> (L.) Holub	Onagraceae	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	982.5
<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

540 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

(Continued on Next Page)

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.

⁵⁴⁴ 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)

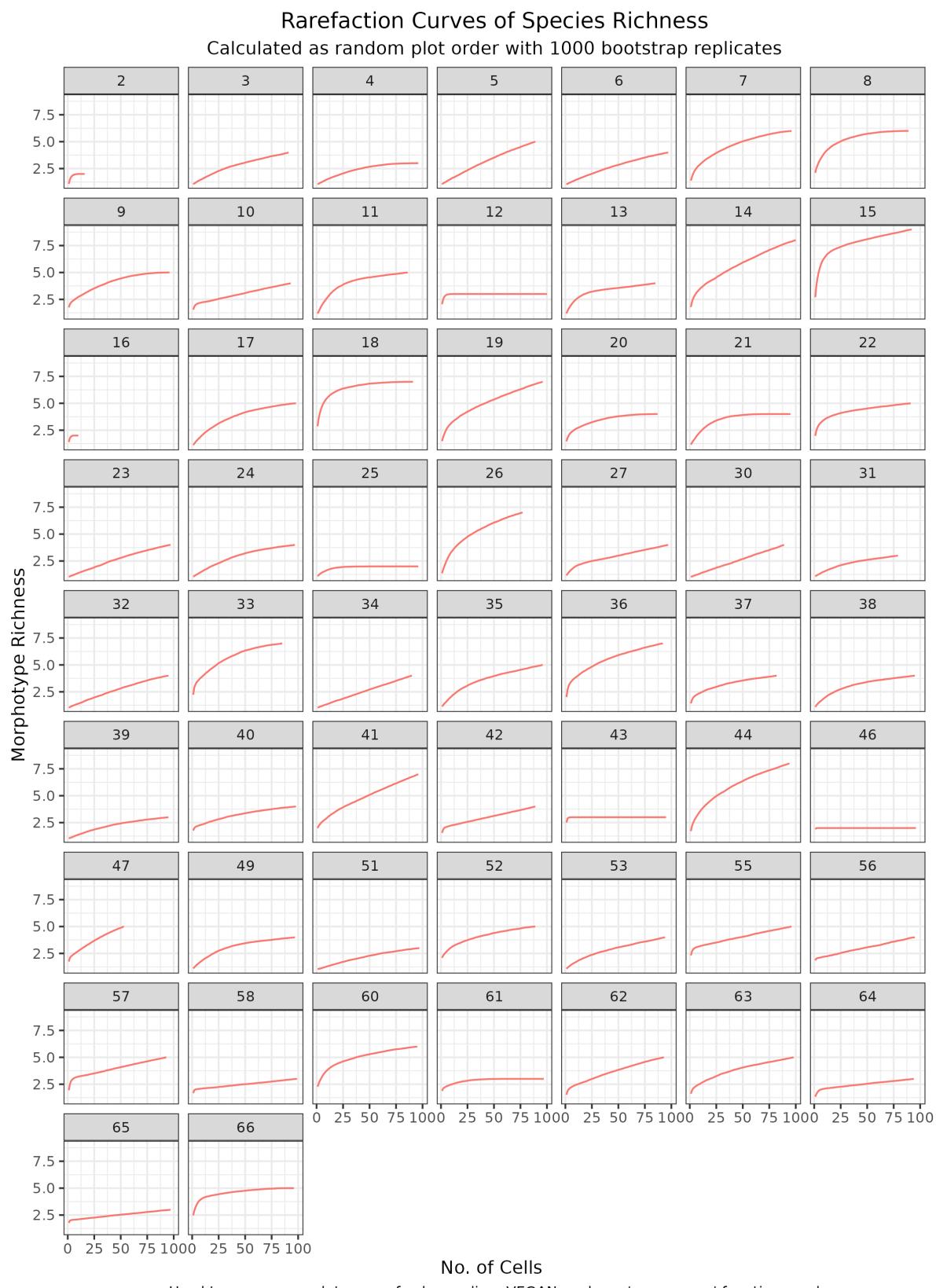
34b: Apertures not annulate – grains appear more or less circular (~ERICACEAE)

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



Species Richness Abundance Estimating via Hill Numbers ($q = 0$)

Confidence Interval of 99% with 1000 Bootstrap replicates

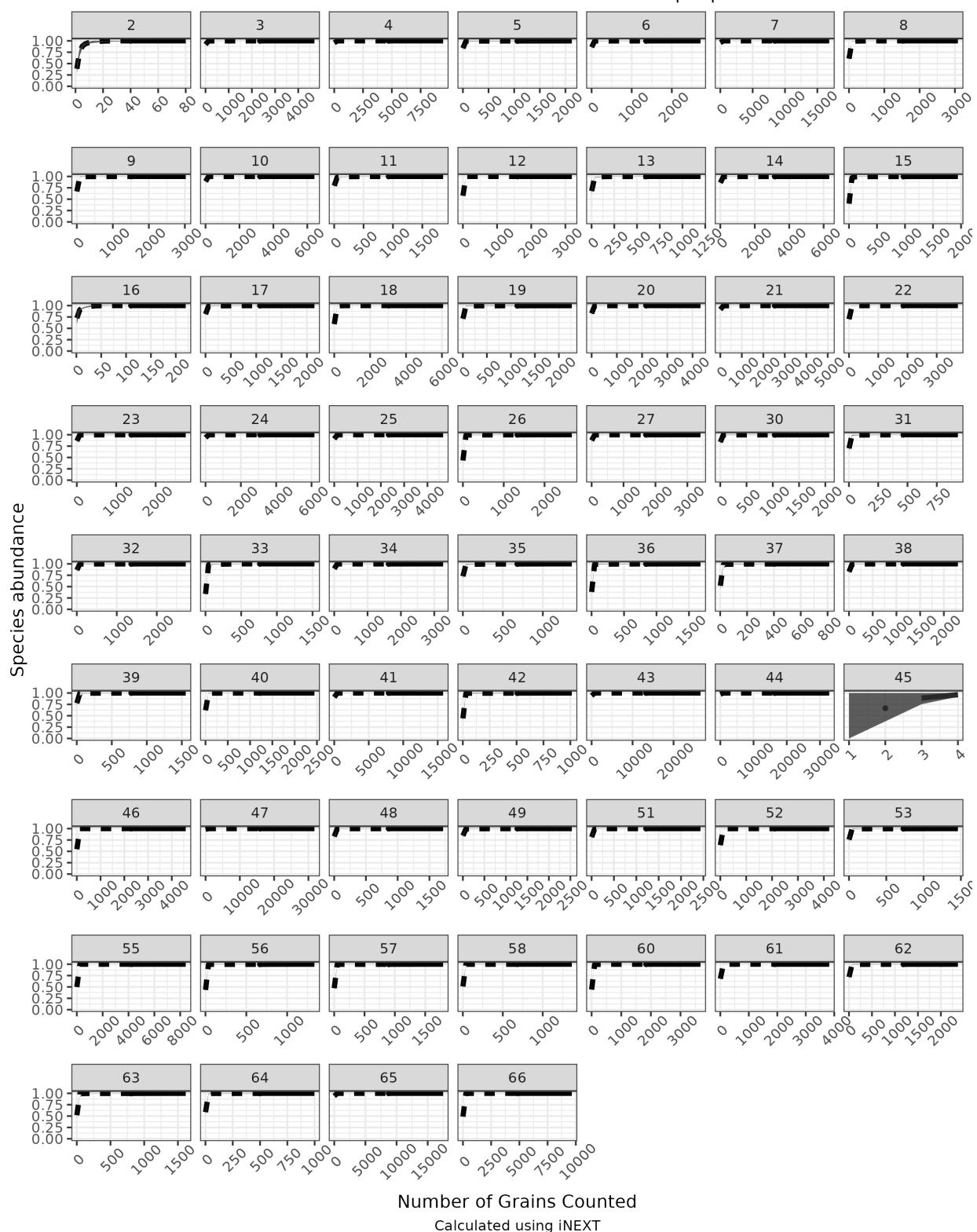


Table 1: All species present in the Reference Sequence Databases
(Kraken and BLAST)

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>Chrysanthmnus greenei</i> <i>Cirsium pannonicum</i> <i>Cirsium parryi</i> <i>Cirsium vulgare</i> <i>Crepis pygmaea</i> <i>Ericameria parryi</i> <i>Erigeron ecuadoriensis</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>

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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>Symphytum 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>
		<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>
	Caryophyllaceae	<i>Nemophila menziesii</i> <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>Polygonaceae</i> <i>Rumex induratus</i> <i>Rumex spinosus</i>
		<i>Celastrales</i> <i>Celastraceae</i> <i>Parnassia faberi</i> <i>Parnassia palustris</i> <i>Paxistima canbyi</i>
Ericales	Ericaceae	<i>Gaultheria prostrata</i> <i>Moneses uniflora</i>
		<i>Orthilia secunda</i> <i>Vaccinium vitis-idaea</i>
	Polemoniaceae	<i>Collomia grandiflora</i> <i>Ipomopsis aggregata</i> <i>Phlox douglasii</i>
		<i>Primulaceae</i> <i>Androsace studiosorum</i> <i>Androsace vitaliana</i>
		<i>Fabales</i> <i>Fabaceae</i> <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>

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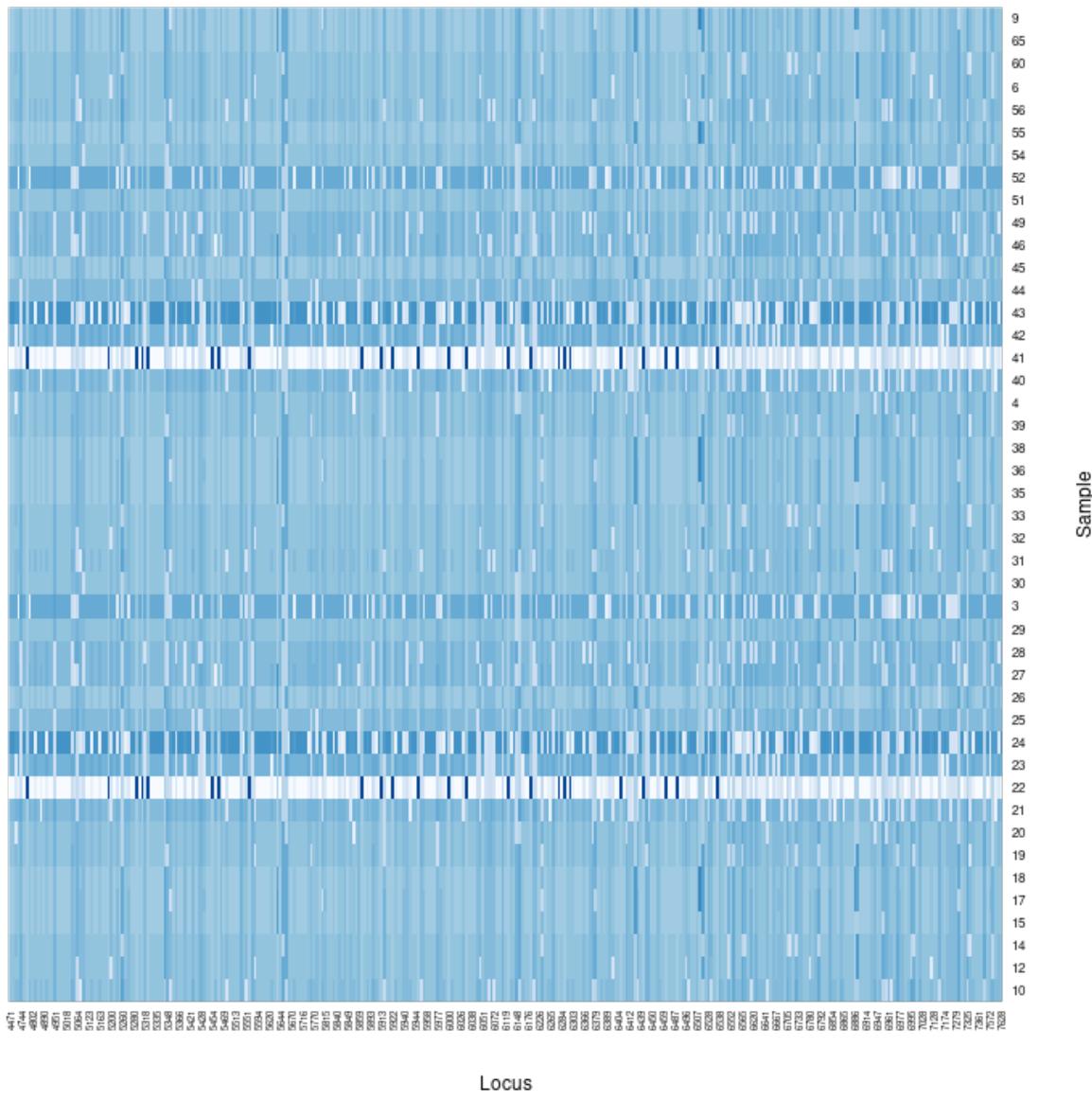
Table 1: All species present in the Reference Sequence Databases
(Kraken and BLAST) (*continued*)

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

x geum* \end{longtable}

559 Appendix XX - Reads Per Loci

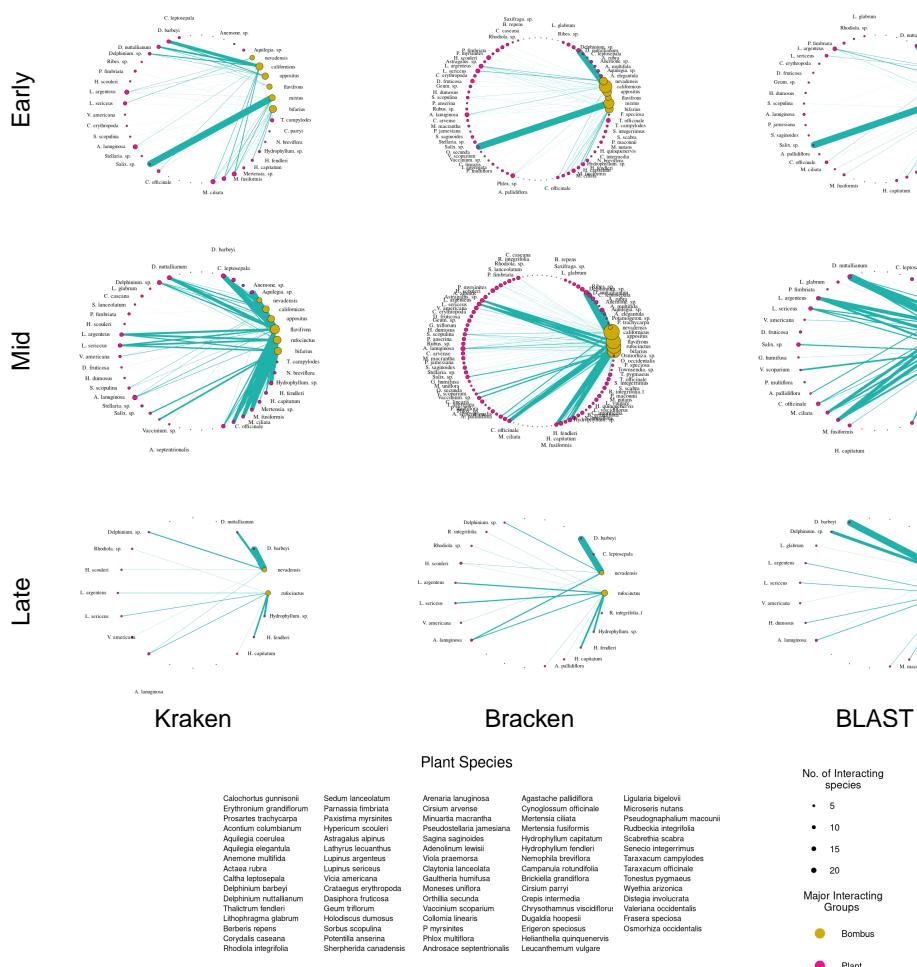
Percent matched reads per locus by sample



560

561 Appendix XX - Comparison of Kraken2, Bracken, and BLAST

Comparision of Foraging Patterns from Three Sequence Alignment Algorithms



563 Appendix XX - Models used for Species Distribution Model Ensembles

564 *Generalised Linear Models (GLM)*

565 *Generalised Additive Models (GAM)*

566 The two machine learning models utilize Ensemble learning.

567 Decision trees, ...

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

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

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

583 Random Forest have high bias and low variance, where boosted regressions trees have low bias and high
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Biotically pollinated plant genera with morphological or molecular data



Figure 1: 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.

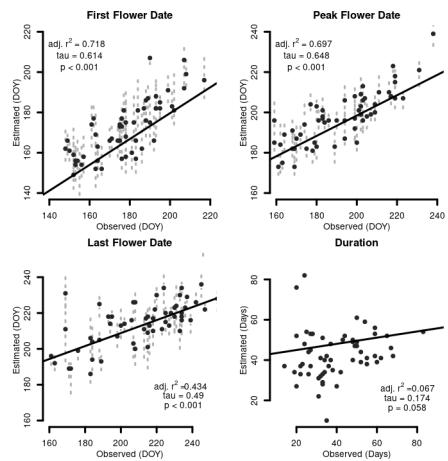


Figure 2: Modelled dates of when major flowering events occurred

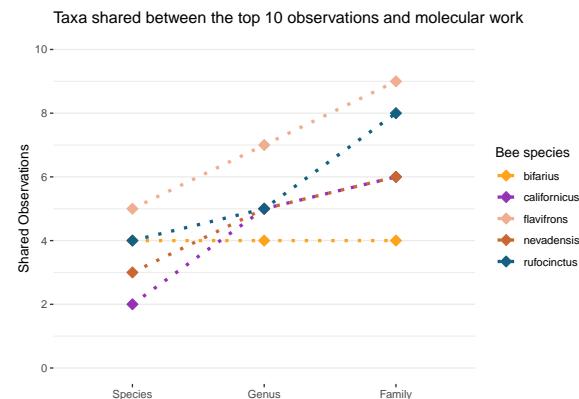


Figure 3: Number of the ten most commonly visited plants which are also in the top ten most common sequences

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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	Absence	25620	3838	11130	1653
	Presence	6614	28248	2758	12024