Semi-quantitative characterisation of mixed pollen samples using MinION sequencing and Reverse Metagenomics (RevMet)

Ned Peel[1,2], Lynn V. Dicks[2], Matthew D. Clark[1,3], Darren Heavens[1], Lawrence Percival-Alwyn[1], Chris Cooper[4], Richard G. Davies[2], Richard M. Leggett[1], Douglas W. Yu[2,5,6,*]

[1] Earlham Institute, Norwich Research Park, Norwich, UK

[2] University of East Anglia, Norwich Research Park, Norwich, UK

[3] Natural History Museum, London, UK

[4] University of Cambridge, Cambridge, UK

[5] State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China

[6] Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming, China

*Corresponding author: Douglas.Yu@uea.ac.uk

Running title: Characterisation of mixed pollen with RevMet

Keywords: bees, diet analysis, genome skim, metabarcoding, metagenomics, MinION, pollen, quantitative

Abstract

1. The ability to identify and quantify the constituent plant species that make up a mixed-species sample of pollen has important applications in ecology, conservation, and agriculture. Recently, metabarcoding protocols have been developed for pollen that can identify constituent plant species, but there are strong reasons to doubt that metabarcoding can accurately quantify their relative abundances. A PCR-free, shotgun metagenomics approach has greater potential for accurately quantifying species relative abundances, but applying metagenomics to eukaryotes is challenging due to low numbers of reference genomes.

2. We have developed a pipeline, RevMet (Reverse Metagenomics), that allows reliable and semi-quantitative characterization of the species composition of mixed-species eukaryote samples, such as bee-collected pollen, without requiring reference genomes. Instead, reference species are represented only by 'genome skims': low-cost, low-coverage, short-read sequence datasets. The skims are mapped to individual long reads sequenced from mixed-species samples using the MinION, a portable nanopore sequencing device, and each long read is uniquely assigned to a plant species.

3. We genome-skimmed 49 wild UK plant species, validated our pipeline with mock DNA mixtures of known composition, and then applied RevMet to pollen loads collected from wild bees. We demonstrate that RevMet can identify plant species present in mixed-species samples at proportions of DNA ≥1%, with few false positives and false negatives, and reliably differentiate species represented by high versus low amounts of DNA in a sample.

4. The RevMet pipeline could readily be adapted to generate semi-quantitative datasets for a wide range of mixed eukaryote samples, which could include characterising diets, quantifying allergenic pollen from air samples, quantifying soil fauna, and identifying the

compositions of algal and diatom communities. Our per-sample costs were £90 per genome skim and £60 per pollen sample, and new versions of sequencers available now will further reduce these costs.

Introduction

Pollination is a key ecosystem service; almost 90% of all flowering plant species, including 75% of food crops (mainly fruits, nuts, and vegetables), rely on animal pollination (Ollerton, Winfree, & Tarrant, 2011; Klein *et al.,* 2007). The benefits of pollinators, and pollinator-dependent plants, also include the production of medicines, biofuels, fibres, and construction materials (Potts *et al.,* 2016). There is growing concern over the decline of wild and domesticated pollinators and the resulting decrease in pollination services and crop production (Potts *et al.,* 2010; Burkle, Marlin, & Knight, 2013). These declines are thought to be caused by multiple threats acting together, including habitat loss, climate change, and the spread of diseases (Vanbergen *et al.,* 2013).

To mitigate drivers of pollinator decline, the Intergovernmental Science - Policy Platform for Biodiversity and Ecosystem Services (IPBES) has suggested three complementary strategies: (1) ecological intensification, which involves boosting agricultural production by increasing the provision of supporting ecological processes such as biotic pest regulation, nutrient cycling, and pollination (Bommarco, Kleijn, & Potts, 2013; Tittonell, 2014); (2) strengthening existing diversified farming systems, including gardens and agroforestry, for the generation of ecosystem functions; and (3) investment in ecological infrastructure, to protect, restore, and connect natural and semi-natural habitats across agricultural landscapes, so that pollinator species can more easily disperse and find nesting and floral resources (IPBES 2016).

However, knowledge gaps limit the effectiveness of these strategies (Wood, Holland, & Goulson, 2015; Dicks *et al.,* 2013). For instance, it is still not clear which plant species are the most valuable food resources and how plant species vary in value across pollinator species, over time, and in different environmental conditions. It is also not well understood whether the addition of floral resources might draw pollinators away from pollinator-dependent crop plants (Morandin & Kremen, 2013), or whether floral enhancement will alter levels of plant-target specialism, at the levels of insect species and of individual insects, resulting in changes in pollination efficiency (Lucas *et al.*, 2018; Morales & Traveset, 2008).

Therefore, a crucial technical challenge for understanding plant-pollinator interactions is a method to identify *and* quantify the species of pollen that are consumed by pollinators. Identifying and quantifying pollen has traditionally been carried out by using light microscopy to distinguish plant species by grain morphology, a labour-intensive technique that requires expert knowledge and lacks discriminatory power at lower taxonomic levels (Long & Krupke, 2016; Khansari *et al*., 2012). In contrast, high-throughput DNA sequencing now allows pollen identification without expert knowledge of pollen morphology and taxonomy.

The currently dominant sequence-based method is metabarcoding, which involves amplifying taxonomically informative marker genes from mixed samples via polymerase chain reaction (PCR) (Ji *et al.*, 2013). The resulting amplification products, known as amplicons, are sequenced, and the reads are assigned to taxonomies by matching against barcode databases, such as the Barcode of Life Data System (Ratnasingham & Hebert, 2007). Notably for plants, there is no single barcode gene that matches the resolving power and universality of 16S rRNA for prokaryotes and Cytochrome Oxidase (CO1) for animals (Hollingsworth, Li, Van Der Bank, & Twyford, 2016). Instead, plant-related barcoding studies rely on a combination of marker genes, which include plastid regions *rbcL* and *matK* and the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (Li *et al.,* 2015; Hollingsworth *et al.,* 2016). Metabarcoding of mixed-species pollen samples can reveal the

presence and absence of constituent plant species (or genera), but there are strong reasons to doubt that metabarcoding can accurately quantify their relative abundances, due to PCR amplification biases and varying copy numbers of barcode loci (Keller *et al.,* 2015; Richardson *et al.,* 2015; Sickel *et al.,* 2015; Bell *et al.,* 2017, 2018; Lamb *et al*. 2018).

In contrast to the targeted sequencing approach of metabarcoding, 'shotgun metagenomics' involves randomly sequencing short stretches of genomic DNA from mixed samples. In standard metagenomics, these short reads ('queries') are mapped to either assembled genomes or to collections of barcode genes ('references'), which creates a requirement for large numbers of reference genomes (Sharpton, 2014) or barcodes, with the latter being very inefficient. Species identification is obtained by first calculating a similarity metric between each short read and each reference sequence (e.g. % identity) and then using an algorithm to assign each short read to the most likely reference sequence (Quince, Walker, Simpson, Loman, & Segata, 2017). The potential key advantages of shotgun metagenomics are that it can avoid the PCR-induced biases seen with metabarcoding, especially if PCR-free library preparation protocols are used (see Nayfach & Pollard, 2016; Jones *et al.,* 2015) and that by sampling across the whole genome, variation in the copy numbers of a few loci is rendered less important. However, the requirement for reference genomes means that most shotgun metagenomics studies focus on prokaryotic organisms, since large numbers of prokaryote reference genomes are available. In contrast, eukaryotes are not well represented in sequence databases and as a result have mostly been neglected in metagenomic studies (Escobar-Zepeda, De León, & Sanchez-Flores, 2015). The low numbers of reference genomes for eukaryotic species is because they are more expensive to sequence and assemble (Gilbert & Dupont, 2011).

Here we demonstrate a metagenomic pipeline for eukaryotes that avoids the need to assemble reference genomes. Instead, each reference species is represented by a 'genome skim,' which is a low-cost, low-coverage, shotgun dataset, i.e. simply a set of short reads.

These sets of short reads are used to identify individual *long reads* from pollen that have been generated by sequencing mixed-species pollen loads with the Oxford Nanopore Technologies' (ONT) MinION, a nanopore sequencing device (for a review of MinION applications and performance, see Leggett & Clark, 2017). Here, we generate reference genome skims for 49 wild UK plant species, and we use them to identify and quantify plant species in two kinds of query samples: mock, mixed-plant-species DNA mixtures of known composition and mixed-species pollen samples collected from wild bees. Since the long reads in each query sample are individually identified, we show that the frequency of long reads assigned to a plant species is a reasonably accurate estimate of that species' biomass frequency in a mixed-species sample. We call this pipeline Reverse Metagenomics, or RevMet, because we map reference sequences to query sequences, which is the reverse of the normal metagenomic protocol.

Methods

Sampling of bees and plant tissue

Sample collection took place in the Pensthorpe Natural Park area (52°49'23"N, 0°53'14"E) of Norfolk, UK, during June and July 2016. Leaf samples were collected from all plant species with open flowers, including grasses and trees, within a 100 m radius of the collection site (n = 49 species). Leaf tissue was preserved on dry ice in the field followed by storage at -80 °C. Foraging wild bees (n = 48: 9 *Apis mellifera*, 27 *Bombus terrestris/lucorum complex*, 12 *Bombus lapidarius*) were collected with hand nets or into falcon tubes directly from flowers and euthanized in falcon tubes containing ethanol-soaked tissue paper. Pollen loads were scraped from bee corbiculae using a mounted needle and stored in absolute ethanol. The plant species on which each bee was foraging when collected was recorded.

Leaf tissue DNA extraction, library preparation, and Illumina sequencing

164

165 Leaf tissue from each of the 49 plant species was disrupted by bead-beating using a 4-mm
166 stainless steel bead with a Qiagen TissueLyser II running at 22.5 Hz for 4 min, rotating the
167 adapter sets after 2 min. DNA was extracted using the DNeasy Plant Kit (Qiagen, Hilden,
168 Germany) following manufacturer's instructions. DNA concentrations were measured on a
169 Qubit 2.0 fluorometer (ThermoFisher, Waltham, USA) using the dsDNA HS assay kit, and
170 fragment size distribution was checked with a Genomic DNA Analysis ScreenTape on the
171 TapeStation 2200 (Agilent, Santa Clara, USA).

172

173 The Earlham Institute (Norwich, UK) applied a modified version of Illumina's Nextera
174 protocol, known as Low Input Transposase Enabled (LITE) protocol (Beier *et al.,* 2017), to
175 generate a separate sequencing library for each leaf sample, targeting an average insert
176 size of 500 bp. The LITE libraries were then pooled based on estimated genome sizes
177 (Supplementary Table S1), obtained from the Royal Botanic Gardens Kew Plant DNA C-
178 values database (Bennett and Leitch, 2012), in order to achieve 0.5x coverage of each
179 species genome. The pooled libraries were sequenced on one lane of Illumina HiSeq 2500
180 in Rapid Run mode (250 bp PE).

181

182 **Construction and sequencing of mock pollen samples**

183

184 DNA from twelve of the 49 plant species were used to construct six mock communities.
185 Each mock was made using 200 ng DNA in total, with species added at different
186 proportions: 0.08% to 45.25% (Table 1). For each mock, technical-replicate pairs were
187 prepared using ONT's (Oxford, UK) Rapid Barcoding Sequencing Kit (SQK-RBK001),
188 following the RBK_9031_v2_revl_09Mar2017 version of the manufacturer's protocol. The 12
189 libraries (six mocks, duplicated) were sequenced on a single MinION R9.5 flow cell (FLO-
190 MIN107).

191

192 **Bee-collected pollen DNA extraction, library preparation, and MinION sequencing**

193

194 After removing storage ethanol from the 48 bee-collected pollen loads, the pollen was
195 disrupted with ca. five 1-mm stainless steel beads for 2 min at 22.5 Hz using a Qiagen
196 TissueLyser II, rotating the adapter sets after 1 min. The pollen samples were resuspended
197 in 600 µl CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 100 mM
198 Tris-HCl pH 8.0), 0.5 µl of β-Mercaptoethanol, 4 µl of proteinase K, and vortexed for 5 s.
199 Following a 1 hr incubation at 55 °C, the tubes were centrifuged for 6 min at 18,000 x g. The
200 ≈500 µl of supernatant was extracted to a clean 1.5 ml tube before an equal volume of
201 chilled (2-8 °C) Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) was added to the lysate.
202 The samples were vortexed for 10 s (5 x 2 s bursts), centrifuged for 5 min at 14,000 x g, and
203 the upper aqueous phase (≈ 420 µl) was extracted by pipette and transferred into a clean
204 1.5 ml tube.

205

206 An equal volume of Agencourt AMPure XP beads was added to each sample, vortexed for
207 20 s (10 x 2 s bursts), and then incubated for 10 min at room temperature. By placing the
208 samples onto a magnetic tube rack for 5 min, the beads were separated from the solution,
209 and the cleared supernatant was removed by aspiration. The beads were washed twice
210 using the following protocol: 1 ml of 80% ethanol was added, incubated at room temperature
211 for 30 s, and then removed, followed by air drying for ≈ 3 min. The magnetic beads were
212 resuspended in 55 µl of EB (Elution Buffer: 10 mM Tris-HCl) and incubated at 37 °C for 10
213 min. The tubes were placed back onto the magnetic rack to bind the beads, and the eluted
214 DNA (≈ 50 µl) was transferred into fresh tubes. A 1 µl aliquot of 1-in-10 diluted Qiagen
215 RNase A was added to each DNA sample before being incubated for 30 min at 37 °C. The
216 concentration of the eluted DNA was assessed using the dsDNA HS assay on a Qubit 2.0
217 fluorometer. To check the DNA for degradation, fragment size distributions were checked
218 with a TapeStation 2200 using the Genomic DNA Analysis ScreenTape.

219

220 Finally, the extracted DNA was prepared and sequenced using the same protocol as used
221 for the DNA mocks above, except that only one library was prepared for each sample.
222 Twelve samples can be multiplexed using the Rapid Barcoding Sequencing Kit; we thus
223 required four flow cells. Due to continuous software upgrades by ONT, the specific software
224 versions of *MinKNOW* varied across runs and is recorded in the final sequence files (fast5
225 format), which are available from the EBI's European Nucleotide Archive (see Data
226 accessibility).
227
228 **Illumina and MinION read pre-processing**
229
230 Duplicate reads were removed from the 49 plant-reference Illumina datasets using *NextClip*
231 *1.3.2* (Leggett, Clavijo, Clissold, Clark, & Caccamo, 2014), and then *cutadapt 1.10* (Martin,
232 2011) was used to trim Illumina adaptors and filter out reads shorter than 100 bp. The
233 resulting unmerged FASTQ files constitute our 49 *reference skims*.
234
235 The MinION datasets from the 12 mocks and the 48 pollen loads were basecalled and
236 demultiplexed with *albacore 2.1.10* (ONT). The resulting FASTQ files were converted to
237 FASTA format. We removed long reads deriving from plant organelles because they are
238 highly conserved across plant species and in pilot tests we observed that mapping to
239 organellar long reads resulted in a higher rate of incorrect assignments than mapping to
240 nuclear long reads (data not shown). NCBI Entrez
241 (https://www.ncbi.nlm.nih.gov/sites/batchentrez) was used to download 2,583 Land Plant
242 organelle genomes, including 1,852 chloroplast, 226 mitochondrial, and 505 plastid
243 genomes. Organelle reads were identified by aligning each of the MinION datasets to the
244 organellar genomes using *minimap2 2.7* (Li, 2018) and removed from the FASTA files. The
245 resulting 60 (= 12 + 48) organelle-filtered FASTA files constitute our mock and pollen *query*
246 datasets, and in the next step, we used the 49 plant reference skims to assign a taxonomy
247 to each long read in the mock and pollen query datasets (Fig. 1c).

248

249 **Taxonomic assignment of mock-sample and bee-collected pollen MinION reads**

250

251 We used *bwa mem 0.7.17* (Li, 2013) to map the Illumina reads from each of the 49
252 reference skims against every individual long read in each of the mock and bee-collected
253 pollen datasets. *SAMtools 1.7* (Li *et al.,* 2009) was used to remove unmapped reads and
254 secondary and supplementary alignments. After SAMtools indexing, the depth of mapping
255 coverage at each long-read position was calculated using the SAMtools *depth* function,
256 followed by the calculation of 'percent coverage' for each long read (using a custom python
257 script), defined as the fraction of nucleotide positions that were mapped to by one or more
258 reference-skim Illumina reads.

259

260 We assigned each long read to the plant species that mapped with the highest percentage
261 coverage, unless the highest percent coverage was <15%, in which case the long read's
262 identity was judged ambiguous and left unassigned. Additionally, for clarity of presentation,
263 we implemented a 1% minimum-abundance filter, removing plant species represented by
264 fewer than 1% of the total assigned long reads in each sample.

265

266 **Reference-skim subsampling**

267

268 To estimate a minimum recommended depth of coverage needed per reference skim, we
269 subsampled one of the genome skims, *Knautia arvensis*, which is a major constituent
270 species in mock mixes MM1 and MM2. We randomly subsampled this skim from its
271 maximum of 0.65x down to 0.05x, in steps of 0.05x using a custom script. For each
272 subsample, the whole pipeline was re-run along with the full reference skims of the other 48
273 plant species. The number of mock reads assigned to *Knautia arvensis*, and the number of
274 unassigned reads, at each level of coverage was recorded. This subsampling was repeated
275 three times (Supplementary Fig. S1).

Network construction

We constructed a pollinator-plant network diagram for the 48 wild-bee pollen samples, using the *bipartite 2.11* package (Dormann, Frund, Bluthgen, & Gruber, 2009) for the *R* statistical language (R Core Team, 2018). For presentational clarity, we only show plant species represented by more than 10% of the assigned reads in each sample.

Results

A reference set of plant genome skims

Low genome-coverage, short-read, shotgun-sequencing datasets ('reference skims') were successfully generated for all 49 plant species (Fig. 1a). After pre-processing, the mean estimated coverage was 0.6x (0.1 to 1x, details in Supplementary Table S1).

Mock DNA mixes

The six mock communities, each with two technical replicates, were sequenced on a MinION. These produced reads with mean length 1914 bp (longest 41,058 bp). After demultiplexing, 88.8% of the reads could be assigned to one of the 12 mock mixes, with the remaining reads left unclassified. Sequences originating from organellar genomes made up between 5.1% (MM4.2) to 10.2% (MM3.2) of the reads in the mocks and were removed. The remaining number of reads per mock ranged from 733 (MM2.1) to 2174 (MM4.1), mean 1347.

Taxonomic assignment of mock-sample MinION reads

304 The 49 reference skims were separately mapped to each long read in each of the 12 mock
305 mixes, and each long read was assigned to the plant species that mapped with the highest
306 percent coverage, or left unassigned if the highest coverage was <15%. In total, 65.5% of
307 the mock reads were assigned to a plant species, with 94.7% of those reads being assigned
308 to a species known to be present in that mock sample. Almost all (93.4%) of the 563 false-
309 positive read assignments were made to one species, *Ranunculus acris*, and all these
310 assignments occurred in the mock samples that contained the very closely related species
311 *Ranunculus repens*. We return to this in the Discussion. The few other false-positive
312 assignments all occurred at a rate of less than 1% of the assigned long reads in their mixes
313 and for presentational clarity are not shown in Fig. 2. The full results are in Table S2.
314
315 All of the plant species that had been added to the mock compositions at proportions ≥1%
316 were detected by our method in at least one of the two replicates, and in general, the
317 technical replicates showed a high level of repeatability (Fig. 2). In two of the mocks, there
318 was one species each (*Lotus corniculatus* in MM2 and *Digitalis purpurea* in MM3*)* that were
319 detected in only one of the two replicates. In these two replicates, *Lotus corniculatus* and
320 *Digitalis purpurea* were expected to be present at only 3.0% and 4.6%, respectively. Both of
321 these species were consistently underrepresented across our mock data sets.
322
323 **Reference-skim subsampling**
324
325 As expected, the larger the reference-skim dataset size for *Knautia arvensis*, the more reads
326 in the MM1 and MM2 mocks were assigned to this species and the fewer reads left
327 unassigned. Importantly, the rate of increase was decelerating (Supplementary Fig. S1);
328 over half of the MinION reads that were assigned to *Knautia arvensis* with a 0.65x genome
329 skim could also be assigned with just a 0.1x skim, even though all the other reference skims
330 in the mapping run were kept at their original sizes.
331

332 **Taxonomic assignment of bee-collected pollen MinION reads**

333

334 The 48 bee-collected pollen loads harvested from the corbiculae of three species, *Apis*
335 *mellifera*, *Bombus terrestris/lucorum* complex, and *Bombus lapidarius*, yielded DNA
336 quantities ranging from 191 to 3750 ng, and all successfully produced libraries,
337 demonstrating that pollen carried by individual bees can provide sufficient DNA for MinION
338 sequencing.

339

340 As with the 12 mocks, each of the reference skims was aligned to each long read in each of
341 the 48 pollen samples, the long reads were either assigned to the plant species achieving
342 the highest percent coverage or left unassigned, and any plant species assigned fewer than
343 1% of the long reads in each bee-collected pollen sample was filtered out (Supplementary
344 Table S3). In total, 49.7% of the long reads were assigned to one of the reference plant
345 species. In 38 of the 48 bees (79.2%), pollen from the plant species on which each bee was
346 captured was found to be present in that bee's pollen load (Supplementary Table S3).

347

348 Each of the 48 pollen loads was found to contain one or two major plant species (defined as
349 read frequency ≥10%) (Fig. 3a). All nine of the *Apis mellifera* pollen loads contained a single
350 major species, whereas 16 of 27 *Bombus terrestris/locorum complex* and 6 of 12 *Bombus*
351 *lapidarius* pollen loads were comprised of two major species (Fig. 3a). These differences in
352 mean number of major species were statistically significant (*Apis mellifera* versus *Bombus*
353 *terrestris/locorum complex* (Welch's t-test, $t = -6.15$, $df = 26$, p-value < 0.0001) and versus
354 *Bombus lapidarius* ($t = -3.32$, $df = 11$, p-value < 0.01)) (Fig. 3b). Another way of visualising
355 the wild-bee results is as a plant-pollinator network graph (Fig. 3c). Overall, 6 of the 49
356 reference plant species were identified as major components in the 48 pollen loads, and the
357 majority of bee-collected pollen samples were dominated by one plant species.

358

359 **Discussion**

360

361 Using light microscopy to identify plant species from pollen requires expert knowledge and is
362 costly when applied to many samples (Khansari *et al.,* 2012). There is a need for a quick
363 and low-cost method that can be scaled to large numbers of pollen samples. Metabarcoding
364 is the current leading candidate, but there are concerns over its discriminatory power at
365 lower taxonomic levels, and there is good reason to believe that metabarcoding does not
366 return reliable quantitative data (Keller *et al.,* 2015; Richardson *et al.,* 2015; Sickel *et al.,*
367 2015; Bell *et al.,* 2017, 2018, Lamb *et al*. 2018). A PCR-free shotgun-metagenomics
368 approach has greater potential for providing reliable quantitative analysis with high power for
369 resolving species. However, applying shotgun metagenomics to eukaryotes is challenging
370 due to the lack of reference genomes (Gilbert & Dupont, 2011). We have developed a
371 metagenomics method that avoids the need for reference genomes. Instead, each reference
372 species is represented by just a low-cost genome skim, and we use a set of such skims to
373 identify individual long reads from pollen samples, produced by the MinION sequencer.

374

375 We evaluated our RevMet pipeline with mock DNA mixtures of known composition and then
376 applied the pipeline to pollen collected from wild bees. Our main findings are:

377

378 1) RevMet can identify plant species present in mixed-species samples at proportions
379 of DNA ≥1%, with few false positives and false negatives, and can reliably
380 differentiate species represented by high versus low amounts of DNA in a sample
381 (Fig. 2, Supplementary Table S2).
382 2) Genome skims with sequence coverage as low as 0.05x can be used for detecting
383 species presence and for estimating relative abundance in terms of DNA mass.
384 Increasing skim coverage increases detection power, at a decelerating rate
385 (Supplementary Fig. S1).

3) Individual pollen loads collected from wild *Apis* and *Bombus* bees yield enough DNA for MinION sequencing (Supplementary Table S3) and generate plausible plant-pollinator networks, as evidenced by the fact that (a) 56.3% of the plant species on which the bees were collected were also the dominant constituent of the corresponding pollen sample (and 79% of plant species on which the bees were collected were detected in the corresponding pollen sample) (Supplementary Table S3), and (b) pollen species richnesses and compositions were more similar within bee species than across bee species (Fig. 3).
4) Our per-plant-species cost of a reference skim was £90, and our per-pollen-sample cost was £61, including DNA extraction, library preparation, and sequencing. Sequencing costs will likely drop further, given the new Illumina NovaSeq and new MinION 'Flongle'.

Semi-quantitative species compositions. – We were able to assign roughly 65% of the mock-mix MinION reads and just under 50% of the pollen-load MinION reads to our reference plant species. Importantly, the frequencies of MinION reads that were assigned to each reference plant species were reliably *'semi-quantitative*,' that is, able to differentiate low- and high-frequency plant species (Fig. 2). Within low- and high-abundance categories, accuracy was lower. For example, in mock sample MM1, *Knautia arvensis*, *Galium verum*, and *Crepis capillaris* were the three high-abundance species (each representing 30.3% of total input DNA mass each), and *Papaver somniferum*, *Anagallis arvensis*, and *Sambucus nigra* were the three low-abundance species (each representing 3.0% of total input DNA mass each). The RevMet pipeline estimated the three high-abundance frequencies at means of 34.0%, 14.7%, and 44.0%, and the three low-abundance species at 1.4%, 3.0%, and 3.0%, respectively (Supplementary Table S2).

There are at least three reasons for the remaining quantitative error. First, although we targeted 0.5x per reference skim, coverage still varied across species (Table S1), resulting

414 in different powers of discrimination, as shown by the experiment with subsamples of
415 *Knautia arvensis* (Supplementary Fig. S1). Fortunately, we found that even very low-depth
416 skims of 0.05x are useful for species detection and are probably still useful for differentiating
417 rare from abundant species (albeit with more error) (Supplementary Fig. S1). Genome sizes
418 are also estimated with error, so it is also helpful that the subsampling experiment suggests
419 that detection power asymptotes with higher sequencing depth (Supplementary Fig. S1),
420 and as sequencing costs fall further, we expect that the most robust protocol will be to target
421 1x coverage.
422
423 Second, very closely related species can generate false positives. Our reference-skim
424 database included six congener pairs, and we included two of the pairs (*Papaver* and
425 *Ranunculus*) in the mock mixes. In the case of *Papaver*, there were no *P. rhoeas* false-
426 positives greater than the 1% minimum-abundance filter in the mocks that contained *P.*
427 *somniferum* (MM1 and MM6) (Supplementary Table S2). In contrast, *Ranunculus acris* was
428 regularly incorrectly assigned to reads in mock mixes that contained the closely related
429 congener *Ranunculus repens*. In fact, almost all the false-positive assignments (93.4%)
430 were to *R. acris*. In retrospect, this result is expected because these two species are not
431 easily differentiated by pollen morphology (Forup & Memmott, 2005), floral morphology, or
432 even DNA barcodes (*rbcL* (99.1% similarity), *matK* (96.9%), *ITS2* (95.5%)). In other words,
433 the RevMet results are correctly telling us that the two *Ranunculus* species are very closely
434 related.
435
436 Third, MinION reads have relatively high error rates of roughly 5 to 10% depending on the
437 flow cell and kit used (Leggett & Clark, 2017). Although this is dropping over time, this error
438 rate unavoidably obscures differences between species (although not enough to confound
439 the two *Papaver* species). We note that one of the advantages of the RevMet approach is
440 that we use both sequence similarity and percent coverage as predictors of species
441 presence (Fig. 1C) . Using sequence similarity alone, we observed several instances of low

442 numbers of mapped reads being given false-positive assignments (data not shown). The
443 percent-coverage filter requires many reference-skim reads to independently identify a
444 species before an assignment is made.
445
446 *Reference-skim cost*. – The RevMet pipeline is relatively low cost. In our study, we
447 generated skims for 49 plant species, with genome sizes ranging from roughly 290 Mb
448 (*Epilobium hirsutum*) to just under 15 Gb (*Sambucus nigra*), targeting 0.5x coverage. All
449 skims were produced on a single lane of Illumina HiSeq 2500 (250 PE) at a mean coverage
450 of 0.57x. The average cost per skim in this study was just under £90, which includes the
451 DNA extraction, LITE library preparation, sequencing, and data QC. The per skim cost will
452 be lower in studies with smaller eukaryotic genomes and should be lower with Illumina's new
453 sequencer, the NovaSeq 6000. Genome assembly projects are likely to produce many such
454 datasets for free download in the future.
455
456 *Long-read MinION cost*. – We used ONT's first iteration of the Rapid Barcoding Kit (RBK-
457 001), which relies on transposase to randomly fragment DNA and simultaneously add
458 barcoded adapters. Longer read lengths have an increased likelihood of accurate species
459 assignment because they carry more sequence information. The two main ways to obtain
460 longer reads with transposase-based preparations are to: (1) increase the ratio of DNA to
461 transposase e.g. by increasing the input material or by heat killing a proportion of the
462 transposase (which also lowers sequencing yields); and (2) use higher molecular weight
463 input DNA. Since the release of RBK-001, ONT's chemistry has evolved, and their Rapid-
464 based kits have seen greater sequencing yields. However, the recommended input for the
465 latest iteration of the Rapid Barcoding Kit (RBK-004) is now higher, 400 ng of DNA per
466 sample. That said, we anticipate that input biomasses similar to those used in this study, 200
467 ng, will still be adequate. Also, even 400 ng is achievable, as 36 of 48 of our wild-bee pollen
468 samples samples yielded >400 ng (Supplementary Table S3). ONT have also recently
469 released the Flongle (~$90), which is a disposable nanopore that allows prolonged reuse of

470 the MinION. Our results suggest that ONT's target yield of 1 Gb per Flongle will be more
471 than enough for multiplexing twelve bee-collected pollen loads, reducing per-sample costs
472 from the £61 in this study to just under £16.
473
474 *Application to pollen collected from wild bees*. – The RevMet pipeline detected consistent
475 differences in the compositions of pollen loads collected by honeybees *Apis mellifera* and by
476 the two bumblebees *Bombus terrestris/lucorum* and *B. lapidarius* (Fig. 3). Importantly,
477 because our data are semi-quantitative, we are able to conclude that even the bumblebees
478 showed fidelity to one plant species (Fig. 3C), a result that would be less reliably concluded
479 from metabarcoding data.
480
481 The RevMet pipeline can readily be applied to a wide range of research questions. Most
482 obviously, ReMet could be used to compare pollination networks across large-scale spatial
483 and biogeographical gradients (Pornon *et al.,* 2016). RevMet could potentially also be used
484 to quantify the degree to which co-attraction of pollinators leads not to benefits of increased
485 pollinator numbers but to loss of pollination service via competition (Carvalheiro *et al.* 2014;
486 Pornon *et al.,* 2016). Outside of pollination ecology, there is potential for semi-quantitative
487 assessments of many other eukaryotic species mixtures, including herbivore diets
488 (Bhattacharyya, Dawson, Hipperson, & Ishtiaq, 2018; Kress, García-Robledo, Uriarte, &
489 Erickson, 2015); plant-fungus interactions (Schröter *et al.,* 2018); allergenic pollen species
490 from air samples (Kraaijeveld *et al.,* 2015); and algal and diatom communities (Keller *et al.,*
491 2015). Furthermore, due to the portability and real-time nature of the MinION platform, the
492 method could be optimised for analysis in the field alongside sample collection.
493
494 **Acknowledgements**
495 This work was supported by the Norwich Research Park Science Links Seed Fund, the
496 BBSRC Norwich Research Park Biosciences Doctoral Training Partnership (grant number
497 BB/M011216/1) and BBSRC Core Strategic Programme Grant BB/CSP17270/1 to the

Earlham Institute. LVD is funded by the Natural Environment Research Council (NE/N014472/1). CC was supported by the J. Arthur Ramsay Fund, administered by the Cambridge University Zoology Department. We are grateful for the support of the Earlham Institute Genomics Pipelines group and the NBI Computing infrastructure for Science (CiS) group. We thank Iain Barr for help in fieldwork and Pensthorpe Natural Park for access.

Authors' contributions

MDC, RML, DWY, LVD, and RGD conceived and designed the study. LVD, RGD, and CC collected the samples. NP, DH, and LP performed the experiments. NP, RML, and DWY analyzed the data. NP and DWY led the writing of the manuscript. All authors gave final approval for publication.

Data accessibility

The Illumina and MinION datasets are available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession PRJEB30946. RevMet scripts are available from https://github.com/nedpeel/RevMet and a tutorial using an example dataset can be found at https://revmet.readthedocs.io/en/latest/.

516 **References**

517

518 Beier, S., Himmelbach, A., Colmsee, C., Zhang, X. Q., Barrero, R. A., Zhang, Q., …
519 Mascher, M. (2017). Construction of a map-based reference genome sequence for barley,
520 Hordeum vulgare L. *Scientific Data*, **4**, 170044.

521

522 Bell, K. L., K. S. Burgess, J. C. Botsch, E. K. Dobbs, T. D. Read, and B. J. Brosi. (2018)
523 Quantitative and qualitative assessment of pollen DNA metabarcoding using constructed
524 species mixtures. *Molecular Ecology* **28**, 431–455.

525

526 Bell, K. L., Fowler, J., Burgess, K. S., Dobbs, E. K., Gruenewald, D., Lawley, B., … Brosi, B.
527 J. (2017). Applying pollen DNA metabarcoding to the study of plant-pollinator interactions.
528 *Applications in plant sciences*, **5**, 1600124.

529

530 Bennett, M., & Leitch, I. (2012). Plant DNA C-values Database. Available at
531 http://data.kew.org/cvalues/

532

533 Bhattacharyya, S., Dawson, D. A., Hipperson, H., & Ishtiaq F. (2018). A diet rich in C3 plants
534 reveals the sensitivity of an alpine mammal to climate change. *Molecular Ecology*, **28**, 250-
535 265.

536

537 Bommarco, R., Kleijn, D., & Potts, S. G. (2013). Ecological intensification: Harnessing
538 ecosystem services for food security. *Trends in Ecology and Evolution*, **28**, 230-238.

539

540 Burkle, L. A., Marlin, J. C., & Knight, T. M. (2013). Plant-pollinator interactions over 120
541 years: Loss of species, co-occurrence, and function. *Science*, **339**, 1611-1615.

542

543 Carvalheiro, L. G., Biesmeijer, J. C., Benadi, G., Fründ, J., Stang, M., Bartomeus, I., …
544 Kunin, W. E. (2014). The potential for indirect effects between co-flowering plants via shared
545 pollinators depends on resource abundance, accessibility and relatedness. *Ecology Letters,*
546 **17**, 1389-1399.

547

548 Dicks, L. V., Abrahams, A., Atkinson, J., Biesmeijer, J., Bourn, N., Brown, C., … Sutherland,
549 W. J. (2013). Identifying key knowledge needs for evidence-based conservation of wild
550 insect pollinators: A collaborative cross-sectoral exercise. *Insect Conservation and Diversity*,
551 **6**, 435-446.

552

Dormann, C. F., Frund, J., Bluthgen, N., & Gruber, B. (2009). Indices, Graphs and Null Models: Analyzing Bipartite Ecological Networks. *The Open Ecology Journal*, **2,** 7-24.

Escobar-Zepeda, A., De León, A. V. P., & Sanchez-Flores, A. (2015). The road to metagenomics: From microbiology to DNA sequencing technologies and bioinformatics. *Frontiers in Genetics*, **6**, 348.

Gilbert, J. A., & Dupont, C. L. (2011). Microbial Metagenomics: Beyond the Genome. *Annual Review of Marine Science*, **3**, 347-371.

Hollingsworth, P. M., Li, D. Z., Van Der Bank, M., & Twyford, A. D. (2016). Telling plant species apart with DNA: From barcodes to genomes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **371**, 20150338.

IPBES. (2016). The assessment report on Pollinators, pollination and food production.

Ji, Y., Ashton, L., Pedley, S. M., Edwards, D. P., Tang, Y., Nakamura, A., … Yu, D. W. (2013). Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecology Letters*, **97**, 1966-1979.

Jones, M. B., Highlander, S. K., Anderson, E. L., Li, W., Dayrit, M., Klitgord, N., … Venter, J. C. (2015). Library preparation methodology can influence genomic and functional predictions in human microbiome research. *Proceedings of the National Academy of Sciences*, **112**, 14024-14029.

Keller, A., Danner, N., Grimmer, G., Ankenbrand, M., von der Ohe, K., von der Ohe, W., … Steffan-Dewenter, I. (2015). Evaluating multiplexed next-generation sequencing as a method in palynology for mixed pollen samples. *Plant Biology*, **17**, 558-566.

Khansari, E., Zarre, S., Alizadeh, K., Attar, F., Aghabeigi, F., & Salmaki, Y. (2012). Pollen morphology of Campanula (Campanulaceae) and allied genera in Iran with special focus on its systematic implication. *Flora: Morphology, Distribution, Functional Ecology of Plants*, **207**, 203-211.

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 303-133.

590

591 Kraaijeveld, K., De Weger, L. A., Ventayol García, M., Buermans, H., Frank, J., Hiemstra, P.
592 S., & Den Dunnen, J. T. (2015). Efficient and sensitive identification and quantification of
593 airborne pollen using next-generation DNA sequencing. *Mol Ecol Resour*, **15**, 8-16.
594

595 Kress, W. J., García-Robledo, C., Uriarte, M., & Erickson, D. L. (2015). DNA barcodes for
596 ecology, evolution, and conservation. *Trends in Ecology & Evolution*, **30**, 25-35.
597

598 Lamb, P.D., Hunter, E., Pinnegar, J.K., Creer, S., Davies, R.G. & Taylor, M.I. (2018). How
599 quantitative is metabarcoding: a meta-analytical approach. *Molecular Ecology* **28**, 420-430
600

601 Leggett, R. M., Clavijo, B. J., Clissold, L., Clark, M. D., & Caccamo, M. (2014). Next clip: An
602 analysis and read preparation tool for nextera long mate pair libraries. *Bioinformatics*, **30**,
603 566-568.
604

605 Leggett, R. M., & Clark, M. D. (2017). A world of opportunities with nanopore sequencing.
606 *Journal of Experimental Botany*, **68**, 5419-5429.
607

608 Li H. (2013). Aligning sequence reads, clone sequences and assembly contigs with bwa-
609 mem. arXiv:1303.3997.
610

611 Li, H. (2018). Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics*, **34**,
612 3094-3100.
613

614 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009).
615 The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, **25**, 2078-2079.
616

617 Li, X., Yang, Y., Henry, R. J., Rossetto, M., Wang, Y., & Chen, S. (2015). Plant DNA
618 barcoding: from gene to genome. *Biological Reviews of the Cambridge Philosophical*
619 *Society*, **90**, 157-166.
620

621 Long, E. Y., & Krupke, C. H. (2016). Non-cultivated plants present a season-long route of
622 pesticide exposure for honey bees. *Nature Communications*, **7**, 11629.
623

624 Lucas, A., Bodger, O., Brosi, B. J., Ford, C. R., Forman, D. W., Greig, C., ... de Vere, N.
625 (2018). Generalisation and specialisation in hoverfly (Syrphidae) grassland pollen transport
626 networks revealed by DNA metabarcoding. *Journal of Animal Ecology*, **87**, 1008-1021.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, **17**, 10.

Morales, C. L., & Traveset, A. (2008). Interspecific pollen transfer: magnitude, prevalence and consequences for plant fitness. *Critical Reviews in Plant Sciences*, **27**, 221–238.

Morandin, L. A., & Kremen, C. (2013). Hedgerow restoration promotes pollinator populations and exports native bees to adjacent fields. *Ecological Applications*, **23**, 829–839.

Nayfach, S., & Pollard, K. S. (2016). Toward Accurate and Quantitative Comparative Metagenomics. *Cell*, **166**, 1103-1116.

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, **120**, 321-326.

Pornon, A., Escaravage, N., Burrus, M., Holota, H., Khimoun, A., Mariette, J., … Vidal, M. (2016). Using metabarcoding to reveal and quantify plant-pollinator interactions. *Scientific Reports*, **6**, 27282.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, **25**, 345-353.

Potts, S. G., Imperatriz-Fonseca, V., Ngo, H. T., Aizen, M. A., Biesmeijer, J. C., Breeze, T. D., … Vanbergen, A. J. (2016). Safeguarding pollinators and their values to human well-being. *Nature*, **540**, 220-229.

Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J., & Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology*, **35**, 833-844.

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Available at http://www.R-project.org/.

Ratnasingham, S., & Hebert, P. D. (2007). BOLD: The Barcode of Life Data System (http://www. barcodinglife.org). *Molecular ecology notes*, **7**, 355-364.

Richardson, R. T., Lin, C. H., Sponsler, D. B., Quijia, J. O., Goodell, K., & Johnson, R. M. (2015). Application of ITS2 Metabarcoding to Determine the Provenance of Pollen Collected by Honey Bees in an Agroecosystem. *Applications in Plant Sciences*, **3**, 1400066.

Schlumbaum A., Tensen M., & Jaenicke-Després V. (2008). Ancient plant DNA in archaeobotany. *Vegetation History and Archaeobotany*, **17**, 233-244.

Schröter, K., Wemheuer, B., Pena, R., Schoning, I., Ehbrecht, M., Schall, P., … Polle, A. (2018). Assembly processes of trophic guilds in the root mycobiome of temperate forests. *Molecular Ecology*, **28**, 348-364.

Sickel, W., Ankenbrand, M. J., Grimmer, G., Holzschuh, A., Härtel, S., Lanzen, J., … Keller, A. (2015). Increased efficiency in identifying mixed pollen samples by meta-barcoding with a dual-indexing approach. *BMC Ecology*, **15**, 20.

Sharpton, T. J. (2014). An introduction to the analysis of shotgun metagenomic data. *Frontiers in Plant Science*, **5**, 209.

Tittonell, P. (2014). Ecological intensification of agriculture-sustainable by nature. *Current Opinion in Environmental Sustainability*, **8**, 53-61.

Vanbergen, A. J., Baude, M., Biesmeijer, J. C., Britton, N. F., Brown, M. J. F., Bryden, J., … Wright, G. A. (2013). Threats to an ecosystem service: Pressures on pollinators. *Frontiers in Ecology and the Environment*, **11**, 251-259.

Wood, T. J., Holland, J. M., & Goulson, D. (2015). Pollinator-friendly management does not increase the diversity of farmland bees and wasps. *Biological Conservation*, **187**, 120-126.

691 **Figure legends and table titles**

692

693 Figure 1. RevMet pipeline overview. a) Low coverage, short-read, reference datasets were
694 generated for 49 wild plant species. b) Bee-collected pollen loads were sequenced on a
695 MinION, generating long read datasets. c) The 49 short-read reference datasets were
696 separately mapped to the long-read pollen datasets, and each pollen read was assigned to
697 the plant species that mapped with the highest percent coverage or was left unassigned if
698 the highest coverage was <15%. d) Binned pollen reads were counted, noise was reduced
699 by implementing a 1% minimum-abundance filter, and then the remaining bin counts were
700 converted to percentages.

701

702 Figure 2. Expected vs observed mock mix compositions. Six mock plant DNA mixes, each
703 with two technical replicates, were sequenced on a MinION and the RevMet method was
704 applied. The first stacked bar of each triplet represents the expected proportions based on
705 input DNA. The second and third bar of each triplet reflect the observed MinION read
706 assignments resulting from this pipeline.

707

708 Figure 3. Bee-collected pollen compositions and plant-pollinator interactions. a) The number
709 of individual pollen loads sequenced from three different species of bee. The proportion of
710 pollen loads that contained a single major plant species are represented by green bars,
711 while those with two major plant species are shown in blue. b) Mean number of plant
712 species per pollen load for each of three different species of bee; ** $p<0.01$, *** $p<0.001$. c)
713 Bipartite plant-pollinator network. The upper bars represent individual pollen loads from
714 three different bee species, *Apis mellifera* (red), *Bombus terrestris/lucorum complex* (blue),
715 and *Bombus lapidarius* (purple). The lower bars (grey) represent plant species. Link width
716 indicates the MinION read proportion of each major plant species within each pollen load.

717

718 Table 1. DNA mock mix compositions.

719

720 Figure S1. Numbers of mock-mix reads assigned to *Knautia arvensis*, and declines in the
721 number of unassigned reads, at different reference-skim coverage levels. The subsampling
722 was repeated three times.

723

724 Table S1. Estimated genome sizes, read counts, and coverage for the genome skim
725 references.

726

727 Table S2. RevMet taxonomic assignments of mock-sample MinION reads.

728

729 Table S3. RevMet taxonomic assignments of bee-collected pollen MinION reads and pollen
730 sample information.

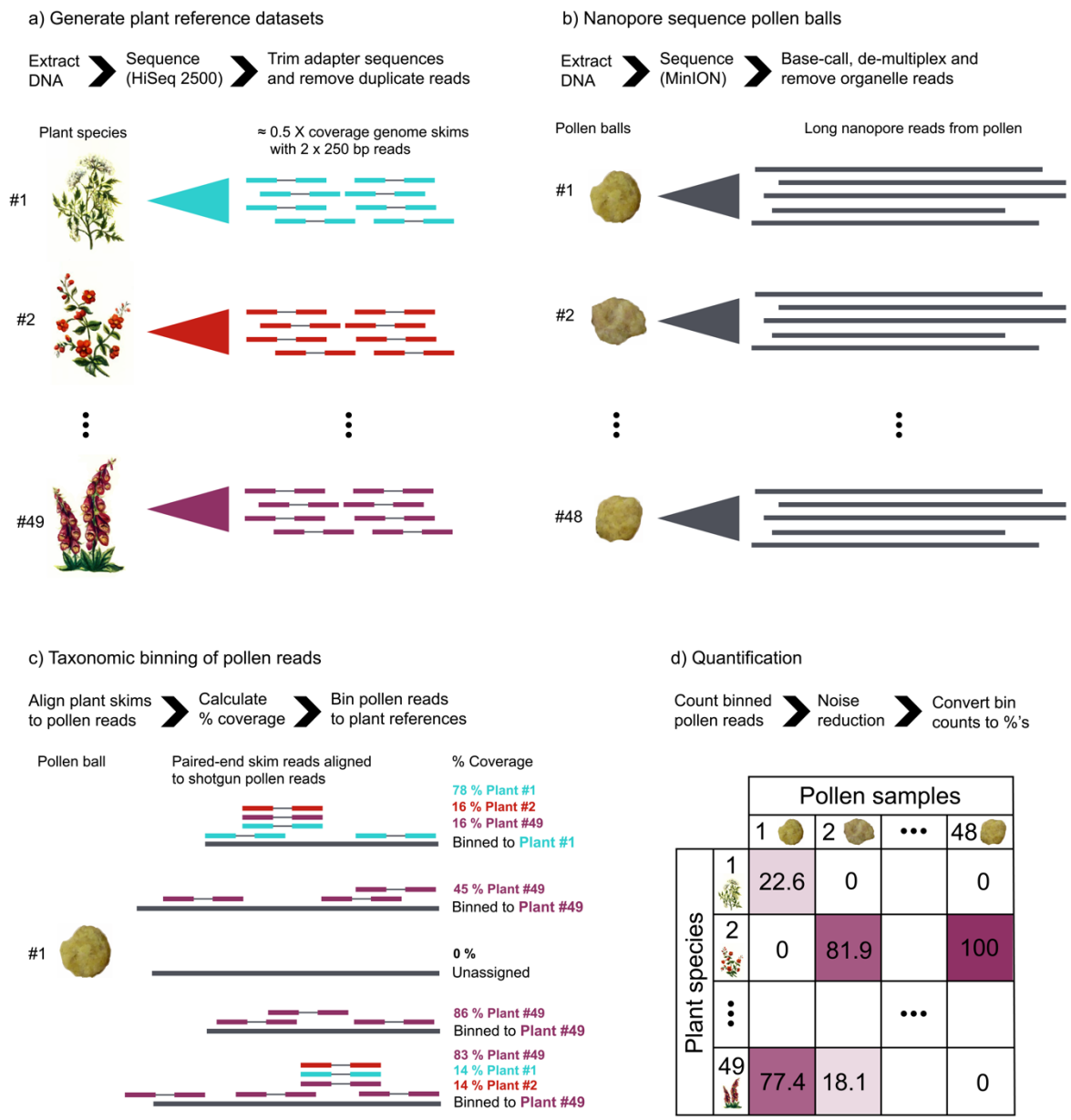

Figure 1. RevMet pipeline overview. a) Low coverage, short-read, reference datasets were generated for 49 wild plant species. b) Bee-collected pollen loads were sequenced on a MinION, generating long read datasets. c) The 49 short-read reference datasets were separately mapped to the long-read pollen datasets, and each pollen read was assigned to the plant species that mapped with the highest percent coverage, or left unassigned if the highest coverage was <15%. d) Binned pollen reads were counted, noise was reduced by implementing a 1% minimum-abundance filter, and then the remaining bin counts were converted to percentages.

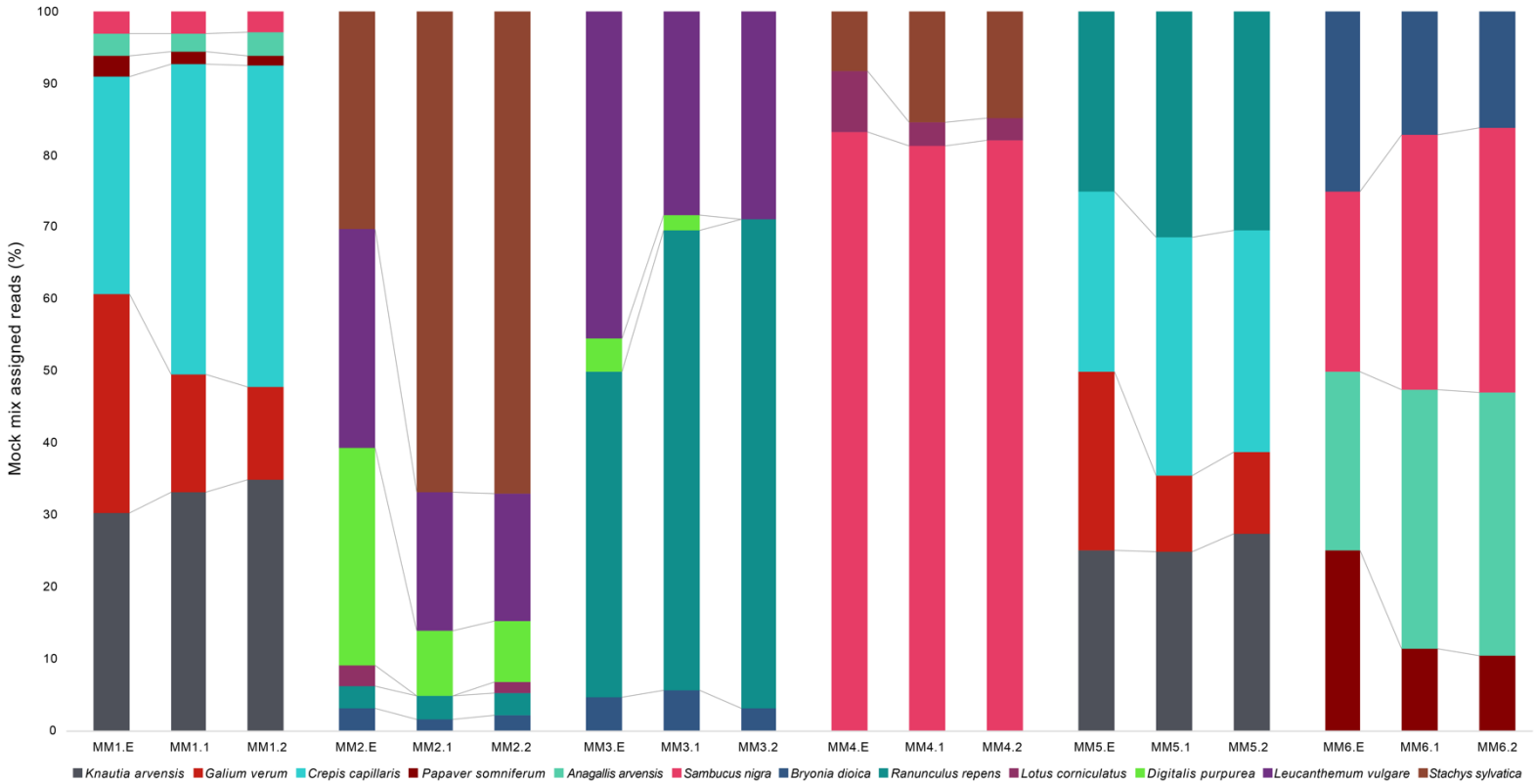

Figure 2. Expected vs observed mock mix compositions. Six mock plant DNA mixes, each with two technical replicates, were sequenced on a MinION and the RevMet method was applied. The first stacked bar of each triplet represents the expected proportions based on input DNA. The second and third bar of each triplet reflect the observed MinION read assignments resulting from this pipeline.

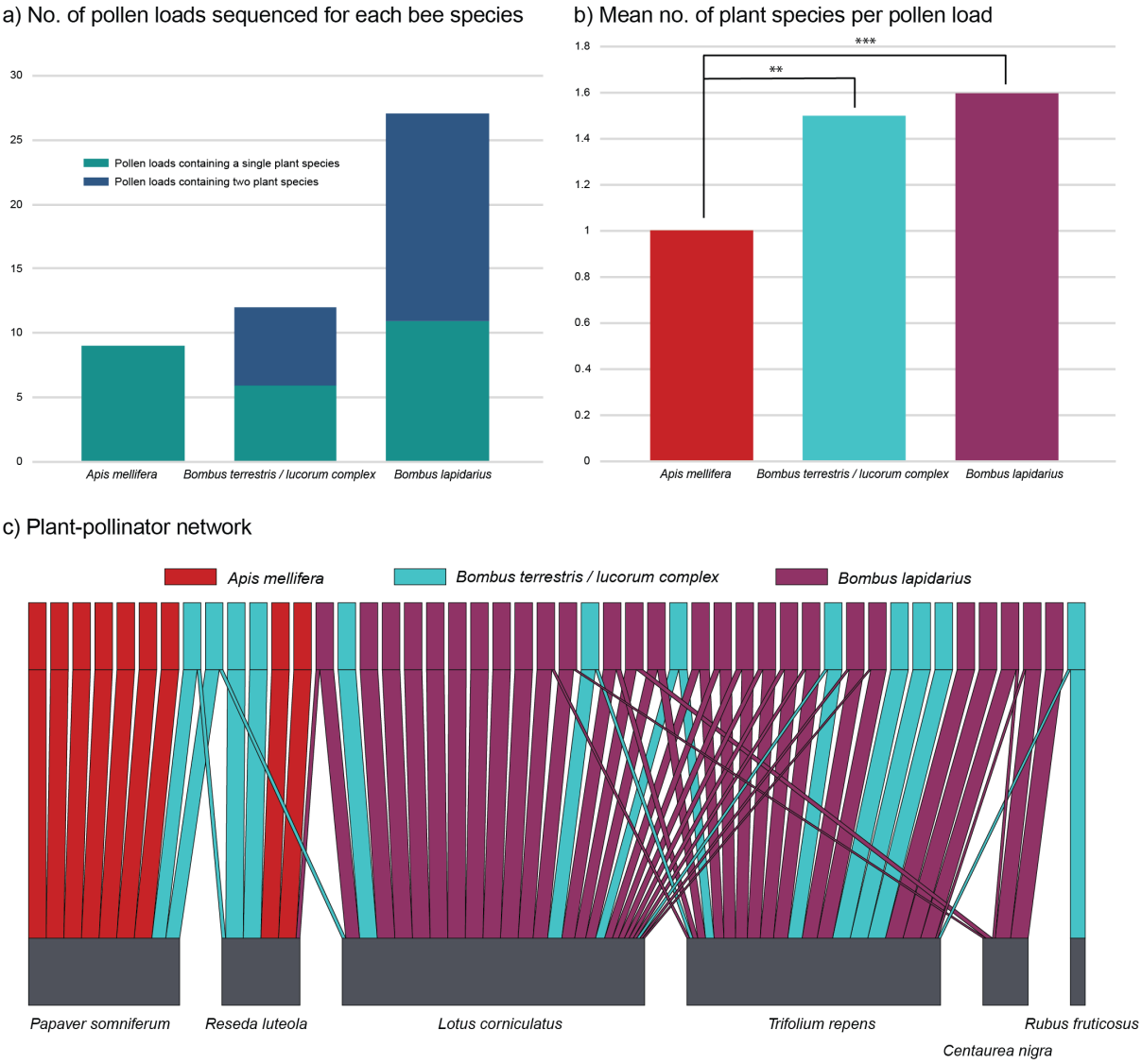

Figure 3. Bee-collected pollen compositions and plant-pollinator interactions. a) The number of individual pollen loads sequenced from three different species of bee. The proportion of pollen loads that contained a single major plant species are represented by green bars, while those with two major plant species are shown in blue. b) Mean number of plant species per pollen load for each of three different species of bee; ** $p<0.01$, *** $p<0.001$. c) Bipartite plant-pollinator network. The upper bars represent individual pollen loads from three different bee species, *Apis mellifera* (red), *Bombus terrestris/lucorum complex* (blue), and *Bombus lapidarius* (purple). The lower bars (grey) represent plant species. Link width indicates the MinION read proportion of each major plant species within each pollen load.

763 **Table 1**. DNA mock community compositions.

764

	Knautia arvensis	*Galium verum*	*Crepis capillaris*	*Papaver somniferum*	*Anagallis arvensis*	*Sambucus nigra*	*Bryonia dioica*	*Ranunculus repens*	*Lotus corniculatus*	*Digitalis purpurea*	*Leucanthemum vulgare*	*Stachys sylvatica*
MM1.Ratios	100	100	100	10	10	10	1	1	1	0	0	0
MM2.Ratios	0	0	0	1	1	1	10	10	10	100	100	100
MM3.Ratios	0	0	0	0	0	0	10	100	0	0	0	0
MM4.Ratios	0	0	0	1	0	1000	0	0	100	0	0	100
MM5.Ratios	100	100	100	0	1	1	1	100	0	0	0	1
MM6.Ratios	1	1	1	100	100	100	100	0	0	0	0	1
MM1.DNA (ng)	60.1	60.1	60.1	6.0	6.0	6.0	0.6	0.6	0.6	0.0	0.0	0.0
MM2.DNA (ng)	0.0	0.0	0.0	0.6	0.6	0.6	6.0	6.0	6.0	60.1	60.1	60.1
MM3.DNA (ng)	0.0	0.0	0.0	0.0	0.0	0.0	9.1	90.5	0.0	9.1	90.5	0.9
MM4.DNA (ng)	0.0	0.0	0.0	0.2	0.0	166.5	0.0	0.0	16.7	0.0	0.0	16.7
MM5.DNA (ng)	49.5	49.5	49.5	0.0	0.5	0.5	0.5	49.5	0.0	0.0	0.0	0.5
MM6.DNA (ng)	0.5	0.5	0.5	49.5	49.5	49.5	49.5	0.0	0.0	0.0	0.0	0.5
MM1.Percentages	30.0%	30.0%	30.0%	3.0%	3.0%	3.0%	0.3%	0.3%	0.3%	0.0%	0.0%	0.0%
MM2.Percentages	0.0%	0.0%	0.0%	0.3%	0.3%	0.3%	3.0%	3.0%	3.0%	30.0%	30.0%	30.0%
MM3.Percentages	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.5%	45.3%	0.0%	4.5%	45.3%	0.5%
MM4.Percentages	0.0%	0.0%	0.0%	0.1%	0.0%	83.3%	0.0%	0.0%	8.3%	0.0%	0.0%	8.3%
MM5.Percentages	24.8%	24.8%	24.8%	0.0%	0.3%	0.3%	0.3%	24.8%	0.0%	0.0%	0.0%	0.3%
MM6.Percentages	0.3%	0.3%	0.3%	24.8%	24.8%	24.8%	24.8%	0.0%	0.0%	0.0%	0.0%	0.3%

765