Nectar Microbial Diversity and Changes Associated with Environmental Exposure

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Nectar Microbial Diversity and Changes Associated with Environmental Exposure.

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Abstract

Background: Plants are critical to global environmental health and food production strategies; most plants utilise flowers as part of their reproduction cycle. Flowers attract pollinators using a range of complex strategies and floral nectar is an essential component of this attraction profile. Nectar is a nutrient rich liquid, containing a range of sugars, organic acids, amino acids, lipids and vitamins, found to be a suitable habitat for a wide range of fungi, but so far, limited bacterial diversity has been detected. Several antimicrobial properties and adverse environmental conditions, such as high osmotic pressure present in the nectar were thought to reduce bacterial numbers.

Results: This study reports the next generation sequencing analysis of the bacterial and fungal diversity in flower nectar. This was achieved in four floral species native to the United Kingdom (*Lamium album*, white dead nettle; *Narcissus pseudonarcissus*, daffodil, *Hyacinthoides non-scripta*, English bluebell and *Digitalis purpurea*, the common foxglove). All flower species examined had a diverse bacterial and fungal populations present with a core microbiome detected, dominated by *Proteobacteria* and *Firmicutes* phyla, while *Basidomycota* were the most persistent fungal phyla in all of the floral nectar types sampled. However, many unique bacterial and fungal species were detected at lower abundances. Furthermore, in *N. pseudonarcissus* and *D. purpurea* floral nectar, the microbial diversity detected in the nectar between flowers exposed to the environment versus non-environment exposed flowers, was different.

Conclusions: These results suggest that floral nectars in different plant species do contain a distinct microbiome and the individual flower microbial community diversity may be affected by floral nectar composition, insect visitation and other environmental factors.

Background

Plants are known to have a wide range of positive and negative interactions with associated microbes [1], and there is a growing recognition of the ecological importance of plants as complex holobionts [2, 3, 4]. There are an increasing number of studies describing the microbial diversity and potential activities in the floral microbiome [5, 6]. This is important because the floral microbiome could modify traits involved in pollinator attraction, and therefore pollinator preference and visitation rate. At present, we are only starting to understand how changes in attractiveness caused by floral nectar microbes modifies pollinator behaviour and the level to which this affects pollination and consequently plant fitness [7, 8].

Nectar is perhaps one of the most important floral traits for the function of pollination, involved in both attracting and rewarding animal pollinators [9, 10, 11]. As nectar is a sugar-rich liquid substance, its microbial diversity probably plays an important role in the mutualism between animal pollinators and flowering plants [12]. Floral nectar contains sucrose, fructose and glucose, which can constitute up to 90% of dry weight and, depending on the plant species, a mixture of organic acids, amino acids, lipids and vitamins [13, 14]. The importance of pollinator attraction was highlighted by a study removing the floral microbiome of *Sambucus nigra* (Elderberry) that resulted in an approximately 65% reduction in floral terpene emissions and a removal of a number of other compounds essential in the volatile profile of nectar [15]. The chemical composition of the nectar also alters the microbial community present [16, 17, 18, 19, 8, 20, 21]. Additionally, pollinator visitation can alter the microbial community present in the flowers as well as the composition and quality of the nectar [22, 23]. Ultimately, changes in nectar induced by the microbial community can affect the strength of the mutualism between flowering plants and pollinators, and even compromise the pollination function [24].

72 Microbial colonisation of nectar may lead to sugar degradation and an increase in bacterial
73 fermentation and secondary products, thought to reduce nectar quality [25, 13]. Because of the
74 potential fitness costs, plants have evolved mechanisms in order to limit or inhibit microbial
75 colonisation [26, 27], including antimicrobial compounds that restrict the growth of certain
76 bacteria [10], production of secondary metabolites such as phenolics [28] and nectarins [29],
77 high osmotic pressure due to the high sugar concentration [30], and the production of reactive
78 oxygen molecules such as hydrogen peroxide [27, 31]. Furthermore, multiple classes of plant
79 secondary compounds have been detected and thought to play a role in preventing or limiting
80 microbial colonisation and growth [32].
81 Despite the range of strategies to prevent microbial colonisation of nectar, the evidence shows
82 that floral nectar contains several species of highly adapted yeast and fungal communities [12,
83 16, 33, 34] with the ability to alter the amino acid and sugar composition of the nectar [16, 21,
84 35]. Bacteria have previously been isolated, cultured and identified in a small number of floral
85 nectar studies [36, 26, 37]. Other studies have described abundant and diverse bacterial
86 communities [38]. The majority of these studies have relied on cultivation methods to examine
87 the microbial communities present [7, 30]. However, due to issues with non-culturability of the
88 majority of microbes in the environment [39, 40], molecular approaches such as microbial
89 community profiling using next generation sequencing may lead to a more comprehensive
90 understanding of the diversity present.
91 In this study, the microbial diversity of four UK native wild flower plant species was assessed
92 using next generation sequencing. Nectar samples were collected from flowers exposed to
93 environmental conditions and pollinators in the field and also flowers of the same plant species
94 kept under controlled conditions in the lab (e.g., not exposed to floral visitors), the bacterial
95 and fungal community was then compared using 16S rRNA genes and ITS sequencing. By

characterising the bacterial and fungal populations present in the nectar we hope to provide a better understanding of the complex community interactions and associations in the floral nectar microbiome.

Materials and Methods

Nectar Sampling.

All plant species selected are commonly found in the wild in the United Kingdom. The plant species included in the study were *Lamium album* (white dead nettle, collected from 50.8290° N, 0.8577° W 28/9/15- 19/5/16), *Narcissus pseudonarcissus* (daffodil wild flowers were collected from 50.8290° N, 0.8577° W collected 22/3/16 - 31/3/16), *Hyacinthoides non-scripta* (English bluebell, collected from 55.9533° N, 3.1883° W 31/5/16) and *Digitalis purpurea* (common foxglove, collected from 50.8290° N, 0.8577° W 7/06/16-27/06/16). Nectar was extracted from open flowers that had been open and exposed to the environment for at least one day (hereafter 'environment exposed' in this study). To examine whether the microbial community changed in flowers exposed to environmental conditions, nectar was extracted from flowers collected as matured buds and brought to the laboratory for nectar extraction once they opened under control conditions (hereafter 'non-environment exposed'). *Narcissus pseudonarcissus* stems generally present a single flower, hence stems with unopened flowers were collected and allowed to open in the laboratory before nectar extraction, allowing 1 day after opening for nectar secretion. As *D. purpurea* presents several flowers per stem, three stems were collected with open flowers and closed flower buds, the environmental samples were collected from all open flowers; the closed buds were allowed to open in the laboratory and the nectar was subsequently collected after 1 day. Samples of *L. album, N. pseudonarcissus, H. non-scripta,* and *D. purpurea,* exposed to environmental conditions were

collected directly in the field, environmental samples were collected from all open flowers and the closed buds for *N. pseudonarcissus,* and *D. purpurea* were collected and allowed to open in the laboratory, the nectar was subsequently collected after 1 day.

For all samples, at time of collection average temperatures ranged from 12-17 °C. Nectar was extracted from the flowers using a 20 µl micro-syringe using aseptic conditions. Nectar extraction was variable for each species and within each flower, with amounts ranging between 0.1 µl- 6 µl per flower. Nectar was extracted from 5 to 15 individual separate flowers (dependent on the volume of nectar extracted) and pooled for each species. Nectar samples were stored briefly at 4°C before being used for culture analysis and DNA extraction.

Analysis of Nectar Sugar Content.

Pooled nectar was used for analysis via refractometer with a total of 3 replicates for each floral species. The total sugar content of the nectar of each floral species was calculated using a refractometer (B&S Eclipse 45) using the Brix % mass sucrose scale [41]. Nonparametric Kruskal-Wallis tests were used to assess the significance (P= 0.05) of differences in nectar sugar composition (*i.e.* % sucrose present) between all environment exposed floral species (environment exposed *N. pseudonarcissus,* environment exposed *D. purpuera, H. non-scripta, L. album*) (n=12) as well as between non-environment exposed samples of the same floral species (*N. pseudonarcissus*, *D. purpurea*) (n=6). Statistical analyses were performed using IBM SPSS v 26.0 [42].

Isolation of Bacteria from Nectar.

An agar was developed for this study based upon the common chemical properties of nectar ([43, 44]; per 1 litre, fructose 0.5 g, sucrose 0.5 g, glucose 0.5 g, soluble starch 0.5 g, yeast extract 0.1 g, dipotassium phosphate 0.3 g, magnesium sulphate 0.05 g (Thermo Scientific, UK), Agar No. 1 (Oxoid) 23g). Nectar was serially diluted in sterile Ringers solution,

144 inoculated onto agar plates and incubated at ~17°C for 10 days in a light dark cycle regimen.
145 Individual colonies were selected from serial dilution plates and sub-cultured in order to obtain
146 pure cultures.

147 **DNA Extraction from Isolates and Nectar.**

148 DNA was extracted directly from nectar using DNeasy Plant Mini Kit (Qiagen, UK) according
149 to manufacturer's instructions. DNA was extracted from isolates using FastDNA SPIN kit for
150 soil (MP Biomedicals, UK) according to manufacturer's instructions. The isolated DNA from
151 both nectar and isolates was stored at -20°C. Universal bacterial primers pA and pH
152 (AGAGTTTGATCCTGGCTCAG; AAGGAGGTGATCCAGCCGCA, [45]) were used in
153 PCR (Biorad T100 Thermocycler), using DreamTaq Green Master Mix (Thermo Scientific,
154 UK) according to manufacturer's instructions. PCR settings; 94°C 5:00, 94°C 0:59, 58°C 0:59,
155 72°C 1:00, Step 2 x 30, 72°C 10:00, 4°C ∞) in order to amplify the 16S rRNA gene from
156 species isolated from nectar. The amplified PCR products were sequenced using Sanger
157 sequencing (Source Bioscience, Nottingham, UK). The resulting sequences were trimmed,
158 edited, checked for quality and identified using BLAST [46].

159 **Analysis of Microbial Community Diversity by Illumina Sequencing.**

160 To profile the microbial community, primers 799F (AACMGGATTAGATACCCKG, [47])
161 and 115R (AGGGTTGCGCTCGTTRC, [48]) were used to amplify the bacterial 16S rRNA
162 V5-V6 gene region and the eukaryotic primers ITS1 and ITS2
163 (TCCGTAGGTGAACCTGCGG; GCTGCGTTCTTCATCGATGC, [49, 50]) were used to
164 amplify the fungal ITS1 region. Sequencing was performed on the Illumina MiSeq V3 platform
165 (LGC Genomics GmbH, Berlin, Germany). Barcode sequences adapters and primer dimer
166 products were removed from the resulting sequence fragments using Illumina bcl2fastq 1.8.4

software and submitted to GenBank (Accession numbers; SRR6433322- SRR6433327 and SRR6433212-SRR6433217).

Bacterial Diversity Analysis.

Sequences of 16S rRNA genes were pre-processing by picking operational taxonomic units (OTUs) using Mothur 1.35.1 [51]. Ambiguous 16S rRNA gene sequences were removed, short alignments filtered out and the remaining sequences aligned using the 16S rRNA gene Mothur-Silva SEED r119 reference. Chimeras were eliminated using the UCHIME algorithm [52] and the Silva reference classification used to classify sequences with OTUs clustered at 97% identity level and annotated with NCBI BLAST+ 2.2.29. Samples were normalised to 8,194, the lowest number of reads per sample for downstream analysis by Quantitative Insights into Microbial Ecology 1.9.0 (Qiime) [53]. Venn diagrams were created using Venny [54] by examining OTUs detected in each sample as described above.

Fungal Diversity Analysis.

Fungal ITS pre-processing and OTU picking was also carried out with Mothur 1.35.1 [51]. Sequences were subsampled in Mothur to 100,000 reads per sample, with distances generated using USEARCH [52]. Chimeras were eliminated using the UCHIME algorithm [52]. The similarity threshold for ITS sequences belonging to the same OTU was set to 97% and clustered by CD-HIT-EST [55] with cluster representative sequences selected based on abundance. Taxonomical classification of OTUs was performed against the UNITE v6 database [56] with species assigned at 97% identity threshold. Samples were normalised to 460, the lowest number of reads per sample for downstream analysis by Qiime [53]. Bacterial and fungal alpha diversity was measured using parallel_alpha_diversity.py script using observed_species and $Chao_1$ metrics [57].

Results

Analysis of Sucrose Content.

Refractometry was used to measure the total (% mass, Brix scale) sucrose present in all nectar types collected (Figure 1). *Lamium album* (30.5%), environment exposed *N. pseudonarcissus* (26%), *H. non-scripta* (22.5%) and environment exposed *D. purpurea* (27.7%), all had similar values (Figure 1). However, the non-environment exposed nectar in *N. pseudonarcissus* and *D. purpurea* had considerably lower values, 2.5% and 4% consecutively (Figure 1). When Kruskal-Wallis analysis was applied to the four different environment exposed floral species (*L. album, N. pseudonarcissus, H. non-scripta, D. purpurea*) significant differences were found (P= 0.021) between % sucrose content. Further, significant differences were observed between both environmental conditions (non-environment exposed and environment exposed) of species *D. purpurea* (P= 0.05) and *N. pseudonarcissus* (P= 0.043).

Bacterial Community Analysis.

Nectar from the four plant species was subjected to 16S rRNA gene sequencing to determine the composition of the bacterial community present. Each nectar type had a distinct microbial community composition (Figure 2A and Figure 4A) with fungal and bacterial sequences being novel and previously undetected in floral nectar. A total of 561,947 sequences, clustering into 528 OTUs (binned at 97% similarity) were obtained from the 16S rRNA gene analysis. Rarefaction analysis based upon lowest number of sequences detected was normalised to 8194, although other samples ranged from 56,871 and 178,495. To estimate bacterial alpha diversity, $Chao_1$ estimates of diversity were applied to OTU distributions, detected up to 8,190 sequences (Table 1). The nectar from *L. album* had the highest observed detectable species richness and *H. non-scripta* the lowest (Figure 1 SI). For all nectar types, differences were detected between

215 S_{obs} and S_{Chao1} and none of the rarefaction analysis reached a stable plateau, indicating that
216 more unique OTUs would be detected with further sequencing analysis (Table 1 and Figure 1
217 SI).

218 **Table 1: Comparison of species richness in different floral species**
219 Comparison of OTU species richness in different floral species using a nonparametric estimate
220 $Chao_1$ [57]. For phylotype richness estimations, OTUs were binned to species.

221

	Fungal ITS analysis		**Bacterial 16S rRNA gene analysis**	
Flower Species	**Observed OTUs**	**$Chao_1$**	**Observed OTUs**	**$Chao_1$**
D. purpurea (Environment exposed)	11	13	65	99
D. purpurea (Non-environment exposed)	8	8	68	83
L. album (Environment exposed)	23	23	75	82
N. pseudonarcissus (Environment exposed)	16	23	31	82
N. pseudonarcissus (Non-environment exposed)	1	1	42	98
H. non-scripta (Environment exposed)	3	3	12	20

222
223

224 Comparing the bacterial communities present in the different floral nectars at the level of
225 family, distinct microbial communities were identified (Figure 2A). Sequences similar to
226 *Proteobacteria* were found to be the most abundant phyla in all of the floral nectar types except
227 for *L. album*, where the most abundant phyla were sequences similar to *Firmicutes* (Figure
228 2A). Overall, the *L. album* nectar had the most 16S rRNA gene diversity detected and was
229 numerically dominated by sequences most similar to *Clostridiaceae*, (49%),

Enterobacteriaceae, genus *Proteus* (10%) and *Chitinophagaceae*, genus *Hydrotalea* (6%) (Figure 2). Environment exposed *D. purpurea* nectar had less detectable bacterial diversity (Figure 1 SI) than the non-environment exposed flower samples with sequences similar to *Acetobacteraceae* (87%), compared to environment exposed flowers which were dominated by *Enterobacteriaceae* (42%), *Acetobacteraceae* (24%), *Clostridiales* family Xl; genus *Clostridium* (18%) and *Enterobacteriaceae*; genus *Erwinia* (14%). Environmentally exposed *N. pseudonarcissus* had higher detectable species richness than the nectar collected in laboratory conditions (Figure 2A). The environment exposed nectar included *Enterobacteraceae*; genus *Erwina* (82%) and *Pseudomonadaceae* (18%), however with nectar collected in the laboratory 99% of sequences were similar to *Enterobacteriaceae*. *H. non-scripta* had the lowest detectable species richness (Figure 2A), with 98% of detectable bacterial diversity from sequences most similar to *Enterobacteriaceae* (98%); genus *Erwinia*.

Comparing environment exposed and non-environment exposed nectar in *N. psuedonarcissus* and *D. purpurea*, a core microbiome was detected (Figure 3) with seventeen orders of bacteria consistently present in both species of flower, in environment exposed and non-exposed conditions. The bacteria consistently detected included *Burkholderiales; Corynebacteriales*; *Micrococcales*; *Bacillales*; *Rhizobiales*; *Lactobacillales*; *Clostridiales*; *Propionibacteriales*; *Sphingobacteriales*; *Rhodospirillales*; *Enterobacteriales* and *Pseudomonadales*.

Fungal Community Analysis.

Using the ITS1 region the fungal community diversity was examined in the nectar of each floral nectar type. In comparison to the bacterial 16S rRNA gene analysis much less diversity was detected, with a total of 250,827 sequences analysed, clustering into 74 OTUs (binned at 97% similarity). The rarefaction curves (Figure 2 SI) indicate that good levels of coverage were

254 achieved for *H. non-scripta* and non-environment exposed *N. pseudonarcissus* but further
255 sequencing would reveal more diversity in the other samples. The $Chao_1$ estimator was applied
256 to OTU distributions (Table 1) to estimate alpha diversity.
257 *Lamium album* displayed the highest detectable fungal species diversity and the environment
258 exposed *N. pseudonarcissus* had the lowest (Figure 2 SI). The greatest difference between S_{obs}
259 and S_{Chao1} was detected in the environment exposed *N. pseudonarcissus,* indicating that more
260 unique OTUs could be detected with further sequencing analysis. Fungal sequences with high
261 sequence similarity to *Basidiomycota* dominated all four plant species. Within this phyla
262 *Tremellomycetes* were observed to be the dominant class, except environment exposed *D.*
263 *purpurea* which was dominated by sequences similar to *Agaricales*.
264 Analysis of fungal genera indicated that environment exposed *D. purpurea* had less detectable
265 diversity than non-environment exposed flowers (Figure 2 SI). The only genera present in both
266 environments, exposed and non-environment exposed, were sequences similar to
267 *Cystofilobasidium*, but these sequences were more numerically dominant in non-environment
268 exposed flowers (Figure 2 SI). Other than *Cystofilobasidium* non-environment exposed *D.*
269 *purpurea* was dominated by *Sporobolomyces* (33%) and *Fusarium* (10%). *Lamium album*
270 flowers were dominated by sequences similar to *Cryptococcus* and had the highest detectable
271 species richness of all floral species tested (Figure 4A). When comparing environment exposed
272 and non-environment exposed *N. pseudonarcissus,* the environment exposed flowers had
273 higher detectable species richness (Figure 4A), being dominated by sequences similar to
274 *Cryptococcus* (49%), *Geastrum* (16%) and *Hyphoderma* (11%), whilst non-environment
275 exposed flowers were solely dominated by sequences similar to *Guehomyces* (100%), and was
276 the only genera to be found in both environment exposed and non-environment exposed

277 flowers. *H. non-scripta* was dominated by sequences similar to *Cystofilobasidium* (81%) and
278 *Guehomyces* (19%).
279
280 **Isolates Obtained from Nectar.**
281 The most abundant culturable bacterial species isolated from the nectar of all four plant species
282 using the nectar agar were sequenced (Table 2). The most numerical dominant culturable
283 isolate in *L. album* nectar was most similar to *Variovorax paradoxus* (Table 2). Nectar of
284 environment exposed *N. pseudonarcissus, D. purpurea* and *H. non-scripta* contained isolates
285 was highly similar to *Pseudomonas fluorescens*, *Curtobacterium flaccumfaciens,*
286 *Obseumbacterium proteus and Gluconobacter oxydans* (Table 2) (Accession Numbers
287 MG552855-MG552861).
288
289 **Table 2: Sequence similarity of bacterial isolates**
290 Sequence similarity of bacterial isolates from the nectar of each floral species.

Host Plant Species	Bacterial Class	Species	% Max Identity	BLAST Accession number
Lamium album	Betaproteobacteria	*Variovorax paradoxus*	98	NC 014931.1
Narcissus pseudonarcissus	Gammaproteobacteria	*Pseudomonas fluorescens*	98	NC_016830.1
		Obesumbacterium proteus	97	NZ_CP014608.1
	Actinobacteria	*Curtobacterium flaccumfaciens*	97	NZ CP014608.1
Hyacinthoides non-scripta	Actinobacteria	*Curtobacterium flaccumfaciens*	98	NZ_APJN01000083.1
Digitalis purpurea	Alphaproteobacteria	*Gluconobacter oxydans*	96	NC 019396.1
	Gammaproteobacteria	*Pseudomonas fluorescens*	99	NC_016830.1

291
292

293 **Discussion**

294 This study provides a profile of the nectar microbiome in four common plants, native to the
295 UK. Profiling of the 16S rRNA gene and ITS gene using NGS has allowed the detection of
296 complex bacterial and fungal communities in each floral nectar type. These populations
297 differed in composition across species and environmental conditions. Sequencing and
298 subsequent $Chao_1$ indices provided evidence for higher bacterial community richness
299 compared to fungi, in all the flower species and conditions observed (Table 1).

300 In examining bacteria detected, except for *L. album* which appears to have a very unique
301 composition, *Proteobacteria* were detected in the highest relative numbers in all floral species
302 (Figure 2) (93% of the diversity in environment exposed *D. purpurea*, 72% in non-environment
303 exposed, 100% in environment exposed *N. pseudonarcissus*, 99% in non-environment exposed
304 *N. pseudonarcissus* and 100% in environment exposed *H. non-scripta*). However, in *L. album,*
305 sequences similar to *Firmicutes* were the dominant phylum (65%), with the highest detectable
306 species richness with dominant sequences being most similar to *Clostridium* (49%), *Proteus*
307 (10%) and *Hydrotalea* (6%). *Hyacinthoides non scripta* had the floral nectar with the lowest
308 detectable species diversity with the majority of OTUs being similar to *Erwinia* (33%). The
309 consistency of detection in nectar types indicates that members of the *Proteobacteria* are
310 common members of these microbiomes. Nectar communities have previously been found to
311 be dominated by *Proteobacteria* [58], also comprising the main phyla in three pollinator
312 exposed floral species (*Borago officinalis, Centaurea cyanus* and *Symphytum officinale*) but
313 not being detected in non-pollinator exposed 'bagged' flowers of the same species [59].

314 There was a detected effect of environmental exposure, on flower nectar composition.
315 N*arcissus pseudonarcissus* environment exposed flowers were found to have an increase in
316 species richness in comparison to non-environment exposed flowers (Figure 2). Non-

317 environment exposed *N. pseudonarcissus* had 99% of all OTU sequences detected were similar
318 to OTUs in the *Enterbacteriaceae* family (99%). The nectar of environment exposed *N.*
319 *pseudonarcissus* was dominated by sequences similar to *Erwinia* (82%) and *Pseudomonas*
320 (18%). Both *Erwinia* and *Pseudomonas* have been documented in nectar environments [60, 61]
321 with both being the only two genera currently reported to be directly associated with pollinating
322 species acting as bacterial vectors [62]. Similarly, the environment exposed *D. purpurea* was
323 found to have a higher detectable OTU richness in comparison to non-environment exposed *D.*
324 *purpurea* (Figure 2). Environment exposed *D. purpurea* had sequences detected similar to
325 *Erwinia* (14%), *Clostridium sensu stricto* (7%) and *Pseudomonas* (3%), and these genera were
326 also observed in other floral species (*H. non-scripta,* environment exposed *N. pseudonarcissus*
327 and *L. album*). Environmental exposure does appear to alter the bacterial community detected,
328 however, overall, the same phyla (*Proteobacteria* and *Firmicutes*) remain dominant. This
329 dominance observed across differing floral species [37, 14] could suggest the restrictive
330 environmental conditions found in nectar, e.g. high osmotic pressure [30] and presence of
331 hydrogen peroxide, [37] lead to the selection of specifically adapted nectar colonisers.
332 Although there was a clear variation in bacterial communities across plant species,
333 environmental conditions appear to affect community composition, as demonstrated by
334 differences between environment exposed and non-environment exposed samples of the same
335 plant species. However, from Figure 1, it can be observed that nectar collected under
336 environmental conditions had much higher % sucrose detected than the samples collected in
337 laboratory conditions. It could be possible that flowers of these species may take longer to
338 produce nectar with a higher sucrose content than the time that was allowed before collection.
339 Thus, the observed changes in microbial community composition between environment

exposed and non-environment exposed species could be due to these reductions in sucrose content of nectar and further analysis needs to be performed in order to investigate this.

Detected fungal diversity included sequences similar to *Tremellomycetes* (of the phylum *Basidiomycota*) which were found to be dominant in all species, except environment exposed *D. purpurea* which was dominated by *Agaricales* (*Basidiomycota*). Sequences similar to *Tremellomycetes* accounted for the majority of diversity in non-environment exposed and environment exposed *N. pseudonarcissus* (100% and 52% respectively), as well as 100% detected in *H. non-scripta*, 83% in *L. album*, and 42% in non-environment exposed *D. purpurea*. Analysed at the level of genera, *L. album* was dominated by species similar to *Cryptococcus* (80%) which has previously been identified in nectar [63, 64, 65]. In the present study similar sequences were only detected in one other species studied (*N. pseudonarcissus*, environment exposed). The majority of the diversity in *H. non-scripta* was made up of sequences similar to *Cysofilobasidium* (78%) and *Guehomyces* (19%). Similarly to the bacterial species richness, *L. album* was observed to have the highest detectable fungal species richness and *H. non-scripta* the lowest. Environment exposed *N. pseudonarcissus* was found to have a higher detectable fungal species richness in comparison with non-environment exposed *N. pseudonarcissus*. The nectar of the environment exposed flower was dominated by sequences similar to *Cryptococcus* (50%), *Geartrum* (16%) and *Hyphoderma* (11%), whilst non-environment exposed flowers were almost completely dominated by *Geotomyces* (99.9%). This was the sole genera present in both variations of *N. pseudonarcissus,* but present in much higher numbers in non-environment exposed *N. pseudonarcissus*. These findings, along with the bacterial community analysis, further support the idea that species richness identified in the nectar changed not across the plant species studied, but also by the environmental conditions to which the flowers are exposed.

These results show a core microbiome exists within nectar in terms of both bacterial and fungal communities, with 100% of samples containing sequences similar to *Pseudomonadaceae* and *Enterobacteriaceae* and 80% of samples containing sequences similar to *Tremellomycetes*, *Cystofilobasidium,* and *Guehomyces*. Further work should seek to fully assess the diversity and species richness of microbial communities within floral nectar of plants as well as the effect of environmental conditions upon this diversity. For example, differences in the nectar microbial community among plant species could be influenced by the identity of floral visitor and pollinator type [66]. However, the sequence analysis using $Chao_1$ indices suggest additional sampling would be required to provide total coverage specifically of the microbial community present. Additionally, it would be beneficial to assess this diversity utilising nectar extracted from single flowers producing larger amounts of nectar, as in this study pooled nectar of multiple flowers (due to the low amounts of nectar extracted from single flowers) may de-emphasise the full effects of environmental conditions.

Conclusion

This study demonstrates that four UK native plant species have a diverse resident microbial community. While microbial composition was found to vary on a floral species to species basis as proposed by Fridman *et al.* [14], a core microbiome of floral nectar could be detected. *Proteobacteria* and *Firmicutes* were consistently detected in high abundance, as seen in several previous studies [37, 14]. However, this is the first study to examine bacterial and fungal communities in floral nectar exposed to environmental factors and compared to nectar from flowers that opened in controlled conditions in the laboratory. From the considerable differences detected in the fungal and bacterial diversities, environmental conditions (most likely pollinator visitation) may play a considerable role in shaping the microbial community

present [66, 67]. Understanding the microbial diversity present in nectar is essential to assessing their effects on the nutritional value of the nectar, how they strengthen or weaken the plant-pollinator ecological mutualism, and therefore their effects on the efficacy of pollination [8, 68]. Further work should seek to fully assess the diversity and species richness of microbial communities within the floral nectar of other plant species as well as the role of bacteria within the yeast-plant-pollinator system and the effects of the environment of these plants upon diversity.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Raw gene sequences were deposited in the GenBank database; SRR6433322- SRR6433327 and SRR6433212-SRR6433217 and isolate 16S rRNA gene sequences; MG552855-MG552861.

Competing interests

JW is Editor in Chief of Environmental Microbiome – this was disclosed in the cover letter with the submission to enable blind review. All other authors declare no competing interests.

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Authors' contributions

JW and RPB conceived the idea of the study. JW and CL designed experimental work. CL carried out collection of experimental data for *L. album, N. pseudonarcissus, H. non-scripta*. AE carried out collection of experimental data for *D. purpurea.* JW, CM, and CL analysed and interpreted data. RPB carried out statistical analysis of data. JW, CL, CM and RPB drafted manuscript. JW, CL, CM, RPB and AE approved final manuscript.

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Supplementary Information

Supplementary information accompanies this paper

Additional file Figure1 SI. Bacterial rarefaction graphs with OTUs derived from sequencing of V3 region, binned to species. The number of sequences recovered from different floral

species under different conditions, the data was normalised on the sample containing the lowest number of sequences, 8,194 sequences were subsampled in each sample.

Additional file Figure2 SI. Fungal rarefaction graphs with OTUs derived from sequencing of ITS1 region, binned to species. The number of sequences recovered from different floral species under different conditions, the data was normalised on the sample containing the lowest number of sequences, 460 sequences were subsampled in each sample.

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Figure 1: Total % mass sucrose for each floral species

Total % mass sucrose for each floral species measured using the Brix scale via refractometry for each floral species. Hatched bars indicate floral nectar not exposed to environmental conditions. Error bars show standard deviation of means (n=3).

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Figure 2: Relative abundance and OTU distribution of bacterial families

A) Relative abundance of bacterial families (>1%) detected by sequencing the 16S rRNA gene from different floral nectar in environment exposed and non-exposed conditions. Sequences were assigned to OTUs with over 97% sequence identity. Sequences not similar to any family and sequences <1% at family were assigned to unclassified/Other. B) OTU network showing distribution of all OTUs identified to phylum detected via sequencing the 16S rRNA gene region from samples of floral nectar. Node size indicates the dominance of reads assigned to an OTU and node colour indicates consensus

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Figure 3: Bacterial orders present in nectar of *N. pseudonarcissus* and *D. purpurea*

A comparison of the bacterial orders detected in the nectar of daffodils and foxgloves. OTUs were found consistently within *N. pseudonarcissus* (NP) and *D. purpurea* (DP) nectar in both non-environment exposed nectar (Non-EE) and environmentally exposed (EE) nectar. In the detection of a core microbiome seventeen bacterial classes were detected in all samples

Figure 4: Relative abundance and OTU distribution of fungal genera

A) Relative abundance of fungal genera (>1%) detected by sequencing the ITS1 region from different floral nectar in environment exposed and non-exposed conditions. Sequences were assigned to OTUs with over 97% sequence identity. Sequences not similar to any family and sequences <1% at family were assigned to Unclassified/Other. B) OTU network showing distribution of all OTUs identified to class detected via sequencing the ITS2 rDNA region from samples of floral nectar. Node size indicates the dominance of reads assigned to an OTU and node colour indicates consensus taxonomy.

Figures

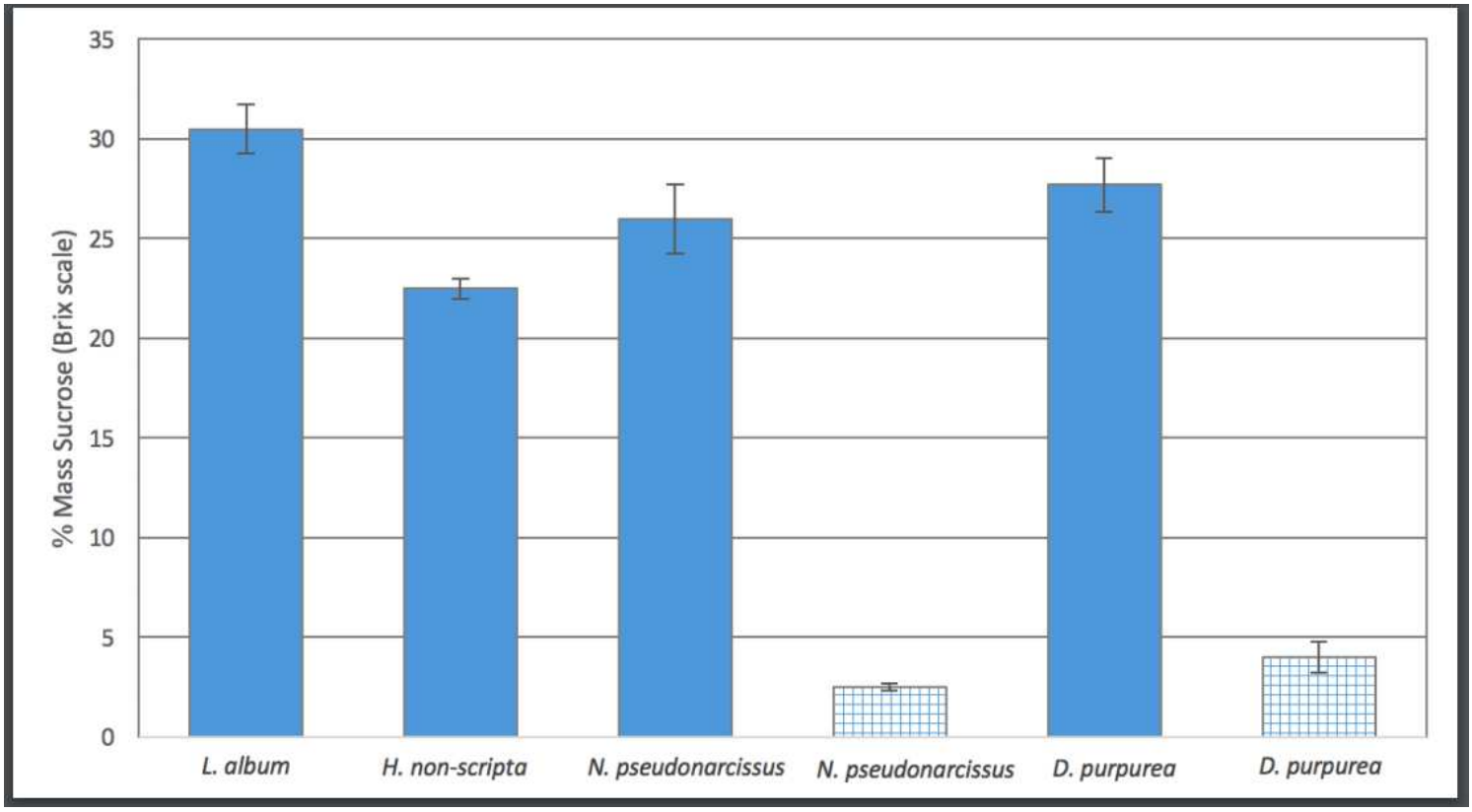

Figure 1

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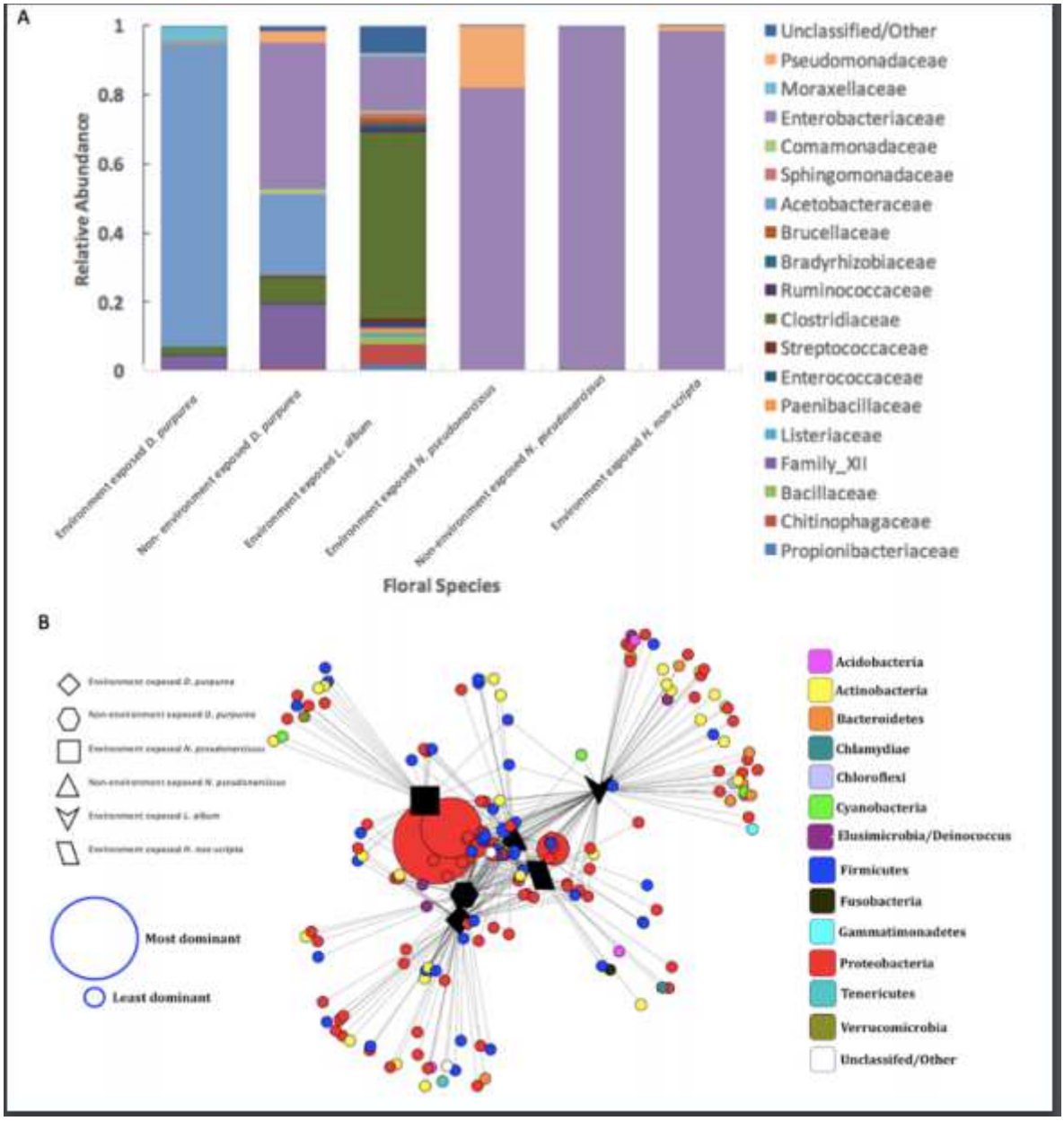

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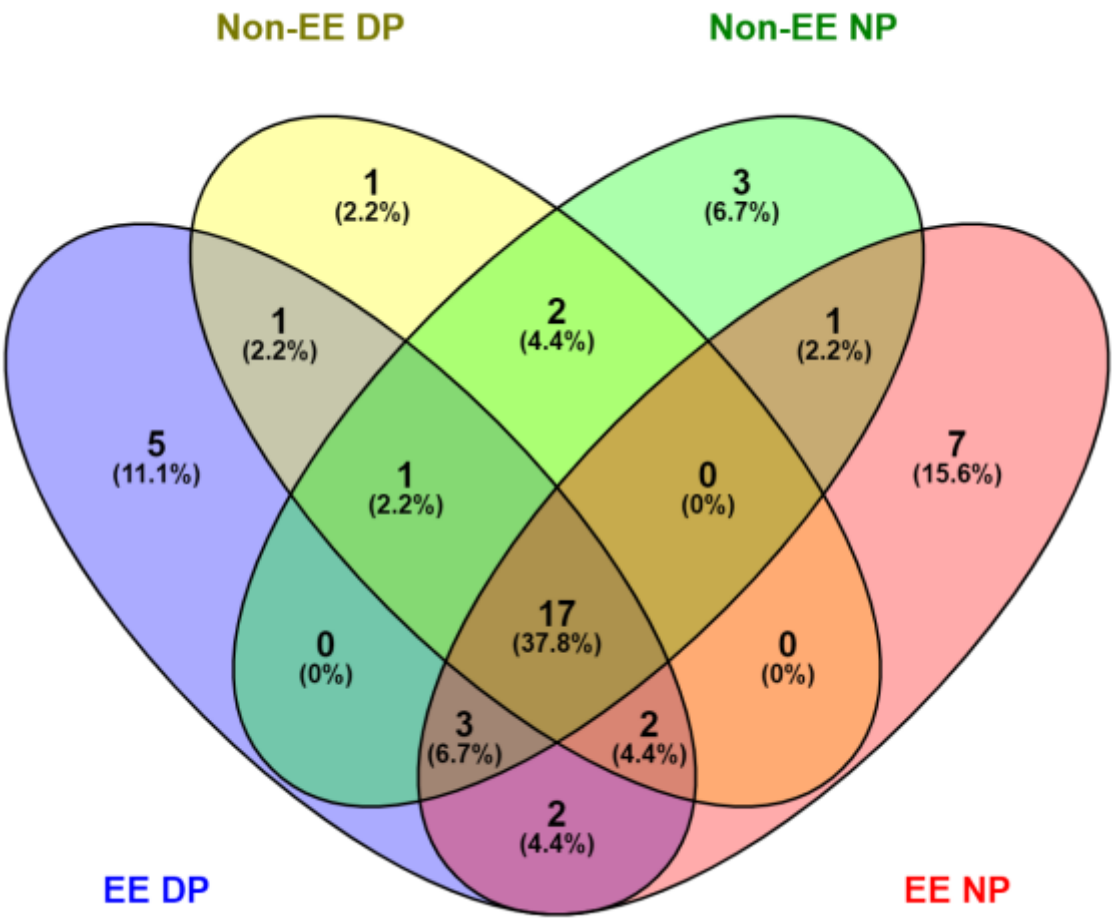
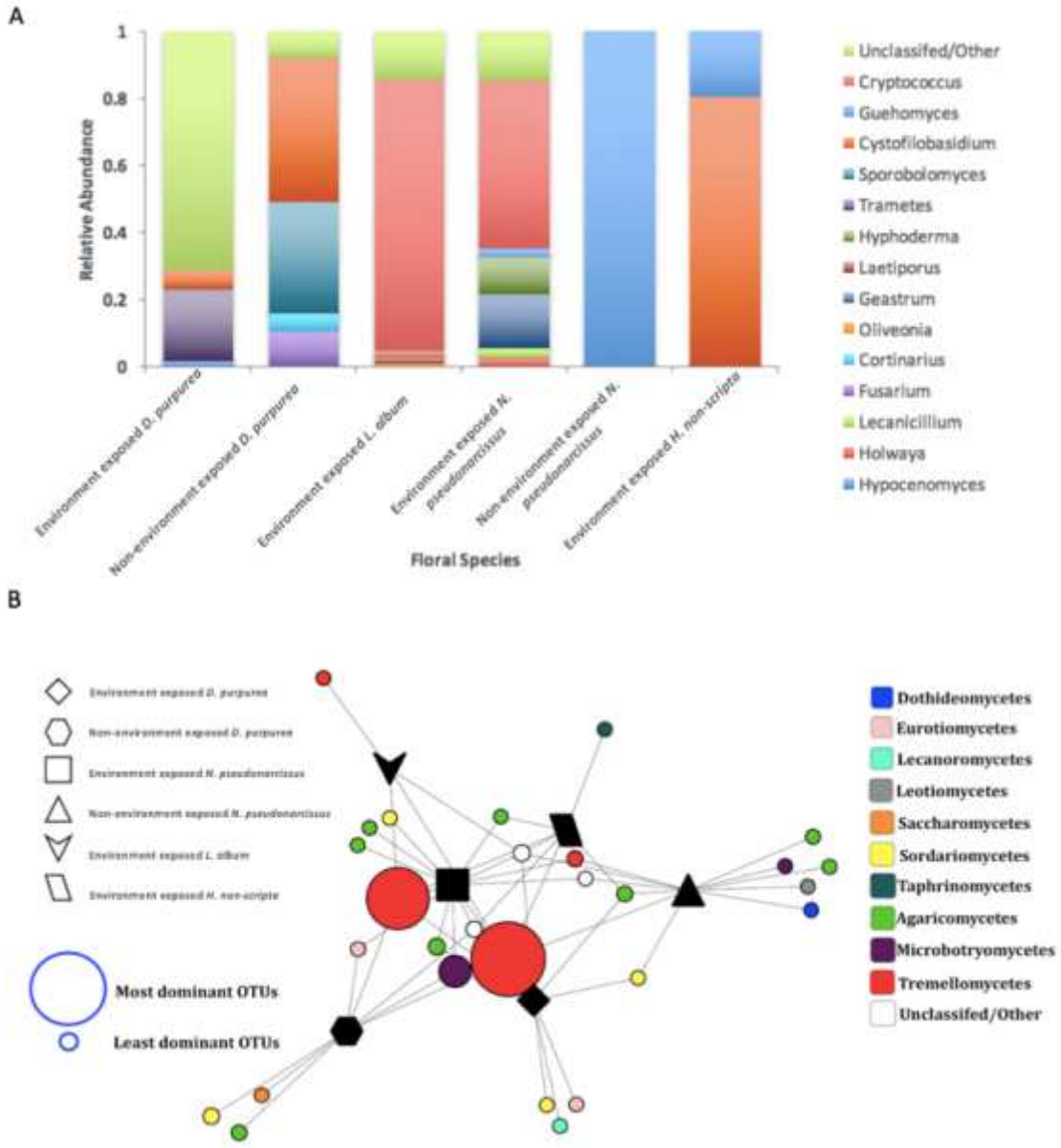


Figure 3

Bacterial orders present in nectar of N. pseudonarcissus and D. purpurea A comparison of the bacterial orders detected in the nectar of daffodils and foxgloves. OTUs were found consistently within N. pseudonarcissus (NP) and D. purpurea (DP) nectar in both non-environment exposed nectar (Non-EE) and environmentally exposed (EE) nectar. In the detection of a core microbiome seventeen bacterial classes were detected in all samples



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Relative abundance and OTU distribution of fungal genera A) Relative abundance of fungal genera (>1%) detected by sequencing the ITS1 region from different floral nectar in environment exposed and non-exposed conditions. Sequences were assigned to OTUs with over 97% sequence identity. Sequences not similar to any family and sequences <1% at family were assigned to Unclassified/Other. B) OTU network showing distribution of all OTUs identified to class detected via sequencing the ITS2 rDNA region from samples of floral nectar. Node size indicates the dominance of reads assigned to an OTU and node colour indicates consensus taxonomy.

Supplementary Files

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- AdditionalfileFigure1SI.pdf
- AdditionalfileFigure2SI.pdf