RESEARCH ARTICLE

Composition, richness and nonrandom assembly of culturable bacterial–microfungal communities in floral nectar of Mediterranean plants

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Abstract

The recent upsurge of interest in the role of floral nectar as a habitat for microorganisms has led to some detailed analyses of nectarivorous yeasts. In contrast, very little is known on the occurrence and diversity of nectar-dwelling bacteria, and bacterial–fungal interactions within nectar remain unexplored. In this work, we studied both the culturable bacteria and microfungi found in the floral nectar of wild Mediterranean plants. In general, bacteria and yeasts were found coexisting in nectar more often than would be expected by chance, and such positive association persisted after accounting for phylogenetic nonindependence of the plant species surveyed. *Metschnikowia* species were confirmed as the main fungal components of nectar communities, and *Acinetobacter* was identified as the main bacterial taxa. Finally, individual Operational Taxonomic Units (OTUs) were found to co-occur less frequently than predicted by random expectations. There existed, however, some pairwise associations between OTUs that seemed to account for the general pattern of positive bacteria–yeasts coexistence. We conclude that the culturable communities of nectar microorganisms associated with wild Mediterranean plants are nonrandom assemblages of bacterial and yeast species.

Introduction

Microorganisms are an essential component of the Earth's biota. They are distributed virtually everywhere, represent the 'unseen' majority of species, individuals and biomass in many environments, and play a central role in the regulation of vital functions that are key to the operation of the biosphere (Whitman *et al.*, 1998; Fry, 2000; Torsvik *et al.*, 2002; Fuhrman, 2009; Konopka, 2009; Fierer & Lennon, 2011). Furthermore, many microbial communities are closely associated with animals and plants, and through direct and indirect effects on their fitness, distribution and population dynamics can mediate many ecological and evolutionary processes (Zilber-Rosenberg & Rosenberg, 2008; Archie & Theis, 2011; Friesen *et al.*, 2011). Despite the global importance of microorganisms, however, current paradigms in ecology and biogeography have been mostly derived from work on plants and animals, and comparatively, little is known about the structure of microbial communities and the forces that control microbial distribution within and between major habitat types (Horner-Devine & Bohannan, 2006; Fierer & Lennon, 2011; Nemergut *et al.*, 2011). This dearth of information is particularly severe in the case of certain underexplored microbial habitats such as floral nectar. Floral nectar is a key component in the mutualism linking angiosperms and animal pollinators (Brandenburg *et al.*, 2009). Owing to the ecological significance of plant pollination in most terrestrial ecosystems, considerable effort has been directed to elucidate the physico-chemical features and nutritional value of floral nectar for pollinators, as well as the physiological processes involved in nectar secretion (see, e.g., Nicolson & Thornburg, 2007; and Heil, 2011 for reviews). In contrast, other fundamental issues in nectar research, such as the role of this floral reward as a microbial habitat, the factors governing the assembly of nectar microbiota and the consequences of multikingdom interactions taking place within and around nectar drops, have started to be explored quite recently.

FEMS MICROBIOLOGY ECOLOGY

Recent work has shown that, irrespective of continent or habitat type, yeasts occur regularly in floral nectar of many plants, where they frequently reach high densities (Brysch-Herzberg, 2004; Herrera *et al.*, 2008, 2009; de Vega *et al.*, 2009; Pozo *et al.*, 2011; Belisle *et al.*, 2012). As a consequence of their metabolic activity, yeasts can profoundly alter nectar chemistry in different ways (e.g. by reducing its total sugar or amino acid content, or releasing ethanol as a fermentation byproduct), which could have an important effect on pollinators' foraging behaviour (Herrera *et al.*, 2008; Wiens *et al.*, 2008; de Vega *et al.*, 2009; Peay *et al.*, 2012; de Vega & Herrera, 2012). Furthermore, nectar yeasts can sometimes inhibit pollen germination (Eisikowitch *et al.*, 1990) and thus potentially interfere with the plant fertilisation process. Yeasts are not, however, the sole microbial inhabitants of floral nectar. Bacteria have been also detected in the nectar of wild (Gilliam *et al.*, 1983; Ehlers & Olesen, 1997; de Vega *et al.*, 2009; Álvarez-Pérez *et al.*, 2012a) and cultivated (Gilliam *et al.*, 1983; Fridman *et al.*, 2012) plants, yet there is almost no information to date on their patterns of distributions across plant species and their possible ecological significance. Two recent studies have shown, for example, that bacteria are common inhabitants of floral nectar in wild South African animal-pollinated plants and some cultivated plants in the Mediterranean Basin (Álvarez-Pérez *et al.*, 2012a; Fridman *et al.*, 2012). Nectar bacterial communities studied so far seem to be characterised by low species richness and phylogenetic diversity in comparison with those associated with other plant substrates (Álvarez-Pérez *et al.*, 2012a; Fridman *et al.*, 2012), as found also for nectar yeast communities (Pozo *et al.*, 2011; Belisle *et al.*, 2012). These parallel findings suggest that, despite being a widespread resource exploited by a broad range of macroorganisms (Wäckers *et al.*, 2007), antimicrobial defences and other limiting factors (Nicolson & Thornburg, 2007) possibly constrain the exploitation of floral nectar by both prokaryotic and eukaryotic microorganisms, which would ultimately favour the evolution of specialisation and nonrandom assembly of nectar-dwelling microbiota. Recent studies support this interpretation for yeasts (Herrera *et al.*, 2010; Peay *et al.*, 2012; Pozo *et al.*, 2012), but relevant information is lacking for bacteria. Furthermore, no previous study has examined patterns of co-occurrence of yeasts and bacteria in nectar in natural plant communities. Given the variety and ecological significance of fungal–bacterial interactions in nature (Wargo & Hogan, 2006; Leveau & Preston, 2008; Frey-Klett *et al.*, 2011), we hypothesise that interactions between these two groups of microorganisms may represent another driving force leading to nonrandom assembly of nectar microbiota.

The main goal of this study was to explore the preceding hypothesis by simultaneously studying the culturable bacterial and yeast communities found in floral nectar. We first described the general patterns of occurrence and co-occurrence of bacteria and yeasts in nectar from a broad, phylogenetically diverse set of Mediterranean plants. Then, bacterial and yeast isolates recovered from nectar samples from a single site were identified by molecular methods and classified into Operational Taxonomic Units (OTUs). Rarefaction-based estimators were applied to these data to assess the diversity and composition of bacterial and fungal components of nectar-dwelling microbial communities. Finally, OTU co-occurrence patterns were examined using simulation methods.

Materials and methods

Study area and plant species sampled

Field sampling for this study was carried out at eight Mediterranean woodland sites scattered around the Andalusia region, southern Spain (Fig. 1). The nearest and most distant sampling sites were approximately 30 km and 380 km apart, respectively. In total, 1002 floral nectar samples from 44 plant species in 22 families were collected and analysed microbiologically as detailed below. A complete list of species sampled is provided in Table 1, and their family affiliation is shown in Table S1 (Supporting Information). Plant identification was based on standard regional floras (Valdés *et al.*, 1987; Blanca *et al.*, 2009).

Flower collection and nectar extraction

Flowering branches, inflorescences or single flowers of nectar-producing plants were collected during mid January–June 2011, a period that included the peak flowering season at all sampling localities. The main criteria used for including a species in the survey were that individual flowers lasted for > 1 day and produced measurable amounts of nectar within 12 h of collection. Whenever possible, widely spaced individuals from different sites were selected for each species. Collected branches, inflorescences or flowers were carefully placed in plastic jars in a portable cooler until taken indoors and then kept under refrigeration (4 °C) until nectar extraction, which was generally performed within 12 h of collection. Extractions of nectar from individual flowers from different individual plants (mean ± SD = 2.5 ± 1.3 flowers per plant; 8.9 ± 5.4 individuals per species) were conducted using sterile calibrated glass microcapillaries (Fisher Scientific, Madrid, Spain). In the case of *Helleborus foetidus*, nectaries from the same flower (mean ± SD = 3.0 ± 0.6) were sampled separately. Nectar volume was determined by

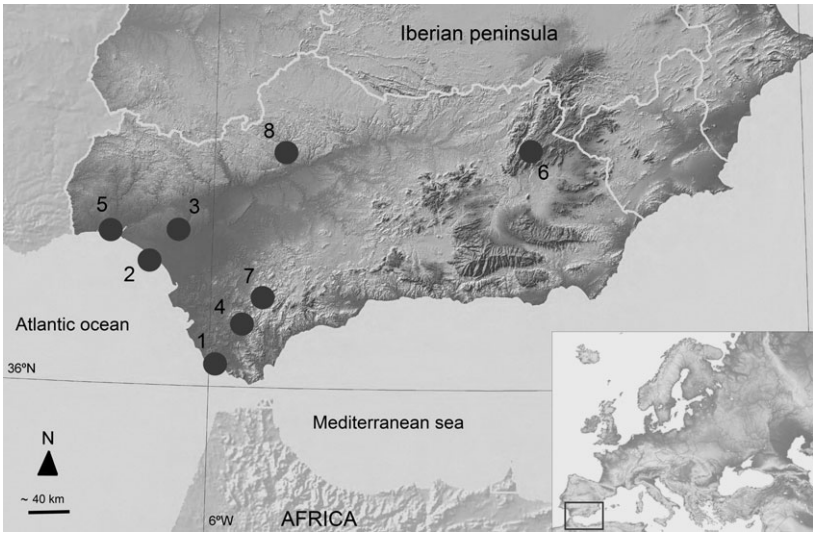

Fig. 1. Distribution of sampling localities used for this study: 1, Barbate (36°11′01″ N, 05°57′31″ W, 100 m.a.s.l.); 2, Cuesta Maneli (37°04′25″ N, 06°41′19″ W, 70 m.a.s.l.); 3, Hinojos (37°18′14″ N, 06°26′15″ W, 90 m.a.s.l.); 4, Los Alcornocales Natural Park (36°33′27″ N, 05°37′35″ W, 165 m.a.s.l.); 5, Punta Umbría (37°15′03″ N, 07°01′14″ W, 7 m.a.s.l.); 6, Sierra de Cazorla (37°54′01″ N, 02°52′31″ W, 1380 m.a.s.l.); 7, Sierra de Grazalema (36°45′23″ N, 05°24′54″ W, 1220 m.a.s.l.); and 8, Sierra de Hornachuelos (37°51′18″ N, 05°16′92″ W, 300 m.a.s.l.).

measuring the length of its column in relation with the total length of the capillar. Between four and 68 nectar samples were examined per species (mean ± SD = 22.8 ± 15.2). Lamiaceae (20.7%), Boraginaceae (12.1%), Plantaginaceae (7.9%) and Fabaceae (7.5%) were the families contributing most nectar samples. Particular care was taken to examine only nectar samples from flowers that were already open and thus had been exposed to pollinator visitation, at the time of collection in the field. Nectar samples were immediately diluted in 500 μL of saline solution (0.85% w/v NaCl; Sigma-Aldrich, Madrid, Spain) to prevent the lysis of microbial cells because of osmotic stress, and stored at 4 °C until microbiological processing.

Microbiological procedures

Twenty-five microlitres of nectar dilutions prepared as described previously was streaked on trypticase soy agar (TSA; Panreac, Castellar del Vallès, Spain) plates. Cultures were incubated at room temperature (*c.* 25 °C) for 7 days. A colony of each phenotypically distinct microbial type was picked and separately subcultivated on TSA to obtain axenic cultures. To minimise the impact of potential negative yeast–bacteria interactions on the recovery rate of these two groups of microorganisms from nectar samples, in those cases when only yeasts were observed on TSA, culturing was repeated on Luria–Bertani (LB) agar (Difco, Sparks, MD) containing 0.1 g L^{-1} of actidione (Sigma-Aldrich) as antifungal agent. On the other hand, when only bacterial colonies were recovered on TSA, culturing was repeated on yeast extract glucose chloramphenicol (YGC, Sigma-Aldrich), which contains 0.1 g L^{-1} of chloramphenicol as antibacterial agent.

Isolates were identified as either bacteria or yeasts from consideration of size, arrangement and other morphological cell features (e.g. presence of budding cells in yeasts). For a subset of 613 samples (61.2% of total) collected from a single locality (site 3 in Fig. 1), the identity of recovered microbial isolates was determined by detailed macro- and microscopic observations, and, in most cases, DNA sequencing and querying public nucleotide databases, as described below. This subset of samples was used in the analyses of community composition and structure. All isolates were stored at −20 °C in LB broth (Difco) containing 25% glycerol (Sigma-Aldrich).

DNA isolation, PCR amplification and partial sequencing of rRNA genes

Genomic DNA was isolated by boiling microbial colonies in 500 μL of ultrapure deionised water at 100 °C for 20 min. Cell debris was removed by centrifugation at 8000 ***g*** for 2 min. For some yeasts isolates, for which DNA extracts obtained by the boiling procedure resulted in poor PCR performance, DNA isolation was repeated using the Qiagen DNeasy Blood and Tissue kit (Qiagen Iberia, Madrid, Spain), according to the manufacturer's instructions.

The bacterial 16S rRNA gene was amplified using primers 27F and 1492R (Lane, 1991). Reaction mixtures and PCR conditions were as described in the study by Álvarez-Pérez *et al.* (2012a). For yeasts isolates, the D1 and D2 domains of the 26S rRNA gene were amplified using primers NL-1/F63 and NL-4/LR3 (Kurtzman & Robnett, 1998; Fell *et al.*, 2000). Reaction mixtures contained 5 μL of NH_4 buffer (10×, Bioline, London, UK),

Table 1. Frequency of occurrence and co-occurrence of bacteria and yeasts in floral nectar of 44 Mediterranean plant species

Plant species (acronym*)	$N^{\dagger}$	Frequency occurrence (% of samples)‡,§		
		Bacteria alone	Bacteria + yeasts	Yeasts alone
Acanthus mollis	21	0.0	4.8	23.8
Anchusa calcarea (AC)	40 (30)	10.0	12.5	12.5*
Antirrhinum graniticum	12	0.0	66.7	0.0**
Antirrhinum major	24	0.0	66.7	8.3***
Armeria gaditana (AG)	26 (26)	0.0	3.9	7.7
Armeria pungens	4	0.0	0.0	0.0
Armeria velutina (AV)	15 (15)	0.0	0.0	13.3
Asphodelus ramosus	39 (21)	2.6	0.0	0.0
Bituminaria bituminosa (BB)	22 (17)	9.1	13.6	4.6*
Campanula rapunculus (CR)	12 (12)	0.0	0.0	0.0
Cerinthe gymnandra (CG)	21 (21)	14.3	0.0	9.5
Convolvulus althaeoides (CA)	28 (28)	21.4	35.7	0.0***
Cynoglossum creticum (CC)	16 (8)	0.0	0.0	0.0
Echium gaditanum (EG)	23 (23)	0.0	13.0	4.4**
Echium plantagineum (EP)	21 (18)	4.8	9.5	0.0*
Erophaca baetica (EB)	17 (17)	0.0	29.4	11.8**
Fedia cornucopiae	8	12.5	0.0	37.5
Fritillaria lusitanica (FL)	10 (10)	30.0	0.0	0.0
Gladiolus illyricus (GI)	28 (28)	7.1	53.6	3.6***
Helleborus foetidus	66	0.0	13.6	86.4
Iris xiphium (IX)	21 (21)	9.5	61.9	0.0***
Lathyrus sp.	9	11.1	0.0	0.0
Lathyrus tingitanus	9	0.0	0.0	0.0
Lavandula stoechas (LS)	68 (28)	1.5	7.4	23.5**
Limodorum abortivum (LA)	14 (14)	0.0	0.0	28.6
Linaria viscosa (LV)	31 (21)	6.5	6.5	9.7
Lonicera implexa (LI)	34 (14)	11.8	55.9	8.8**
Misopates orontium (MO)	12 (12)	0.0	0.0	16.7
Muscari comosum (MC)	21 (21)	0.0	14.3	23.8*
Narcissus longispathus	11	9.1	0.0	63.6
Narcissus papyraceus (NP)	46 (40)	0.0	30.4	30.4***
Orobanche ramosa (OR)	12 (12)	16.7	0.0	8.3
Orobanche rapum-genistae (ORG)	28 (22)	3.6	0.0	0.0
Parentucellia viscosa (PV)	15 (15)	0.0	0.0	0.0
Phlomis purpurea (PP)	47 (9)	0.0	14.9	34.0**
Primula vulgaris	12	8.3	25.0	25.0
Rosmarinus officinalis (RO)	48 (28)	0.0	12.5	18.8***
Scabiosa atropurpurea (SA)	20 (20)	5.0	0.0	5.0
Silene bellidifolia	8	0.0	0.0	0.0
Silene colorata (SC)	10 (10)	0.0	0.0	0.0
Silene nicaeensis	6	0.0	0.0	0.0
Teucrium fruticans (TF)	44 (34)	2.3	20.5	22.7***
Vicia villosa (VV)	18 (18)	0.0	0.0	5.6
Vinca difformis	5	0.0	0.0	0.0
TOTAL	1002 (613)	4.0	15.9	17.8***

*Acronyms are only shown for those plant species for which the identity of microbial isolates was determined by macro- and microscopic observations and DNA sequencing.

†Number of nectar samples analysed per plant species. Figures between parentheses correspond to the number of samples included in the analyses of microbial community composition and structure.

‡Proportion of nectar samples from which only bacteria, only yeasts, or both bacteria and yeasts were isolated in microbiological analyses.

§Asterisks denote levels of statistical significance of bacteria–yeast co-occurrence, tested on the corresponding two-way contingency table: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

1.5 mM $MgCl_2$ (Bioline), 0.4 μM of each primer (Sigma-Aldrich), 100 μM of each dNTP (Sigma-Aldrich), 1.5 U Biotaq DNA polymerase (Bioline) and 2–5 μL of DNA extract in a final volume of 50 μL. Amplification was carried out in a FlexCycler PCR thermal cycler (Analytik Jena, Jena, Germany) and consisted of a denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min.

PCR products were cleaned up with ExoSAP-IT (USB Corporation, Cleveland, OH), which degrades excess primers and nucleotides. Bacterial 16S rRNA gene amplicons were sequenced using the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Madrid, Spain) and the following six primers (Sigma-Aldrich): 27F, 515F, 874F, 519R, 907R and 1492R (Reysenbach *et al.*, 2000; Chanbusarakum & Ullman, 2008). Sequencing reactions of yeast 26S rDNA amplicons were similar, but included NL-1/F63 or NL-4/LR6 oligonucleotides, instead of the aforementioned bacterial primers. In both cases, the sequences were determined on an automated sequencer (ABI Prism 3130xl, Applied Biosystems), and assembled and manually edited with the program SEQUENCHER v.4.9 (Gene Codes Corporation, Inc.,

Ann Arbor, MI). The GenBank accession numbers of the DNA sequences obtained in this study are JX067650–JX067737 and JX067738–JX067815 for bacteria and yeasts, respectively.

Data analyses

Correlation between frequencies of occurrence of bacteria and yeasts

The possible correlation across plant species between frequencies of occurrences of bacteria and yeasts in nectar was explored in the whole set of 44 plant species sampled. Two types of analyses were conducted: (1) simple linear regression using the data of frequencies of occurrence of bacteria and yeasts in floral nectar of each plant species, without taking into account the phylogenetic relations of these latter (TIP analysis) and (2) standardised linear contrasts regression after accounting for the possible influence of phylogenetic correlations present in the data (phylogenetically independent contrasts, PIC analysis; Felsenstein, 1985; Garland *et al.*, 1992). For the PIC analysis, a phylogeny of the set of plant species surveyed was constructed using the Phylomatic tool available in the PHYLOCOM v.4.1 program (Webb & Donoghue, 2005; Webb *et al.*, 2008). The list of surveyed taxa and one of the Angiosperm Phylogeny Group's most recent published phylogenies (the APG3 megatree available at the Phylomatic website: http://svn.phylodiversity.net/tot/megatrees/R20091120.new; last accessed on 6 March 2012) were used as inputs for the analysis. Branch lengths of the phylogenetic tree were obtained by running the BLADJ command in Phylocom and using published data on fossil ages for some of the internal nodes (Wikström *et al.*, 2001) to interpolate the remaining node ages. Computations involving PICs and significance tests were performed with the PDAP:PDTREE module of Mesquite (Midford *et al.*, 2010; Maddison & Maddison, 2011). A number of degrees of freedom equal to the number of polytomies in the aforementioned phylogenetic tree were subtracted to obtain standardised linear contrasts (Purvis & Garland, 1993; Garland & Díaz-Uriarte, 1999).

Taxonomic classification of isolates and delineation of molecular OTUs

Ribosomal RNA gene sequences from bacterial and yeast isolates were compared with reference sequences from the GenBank databases, using Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/), and isolates were assigned to species or the highest taxonomic rank possible.

Nucleotide sequences were included in multiple alignments generated by CLUSTALW (Chenna *et al.*, 2003) separately for bacteria and yeasts. To reduce computational demands, only sequences differing in at least one nucleotide position were included in the alignments. The resulting alignments were trimmed with BIOEDIT v.7.0.9.0 (Hall, 1999) to ensure that all sequences had the same start and end point and analysed with Gblocks (Castresana, 2000) to eliminate ambiguously aligned regions, using 'allowed gap positions = with half', 'minimum length of a block = 5' and default settings for all other options. Determination of the number of distinct OTUs occurring in our sets of DNA sequences and assignment of sequences to OTUs were performed with the program MOTHUR v.1.17.3 (Schloss *et al.*, 2009). Uncorrected pairwise distances between aligned DNA sequences and the average neighbour algorithm were used to define OTUs at the 1% and 3% DNA dissimilarity cut-off values.

Analysis of OTU richness

To assess the overall richness of bacterial and yeast OTUs, rarefaction methods were applied to presence–absence data following two complementary methods (see Pozo *et al.*, 2011 for a similar approach). In the first case, nectar drops were treated as sampling units, and species occurrence data from all nectar samples were analysed together, irrespective of the plant species of origin. This procedure provided 'nectar drop-based' rarefaction curves that assessed overall OTU richness of nectar bacteria and yeasts for the combined multispecific set of plant species surveyed. The second approach for assessing OTU richness was based on the hypothesis that differences between plant species in life style, floral traits and/or nectar chemistry could influence their nectar microbiota. The different host plant species were treated separately, data from all nectar samples from the same plant species were pooled into a single sample, and 'plant species-based' rarefaction curves were thus obtained. Drop-based and plant species-based average rarefaction curves for bacterial and yeast communities were computed with the ESTIMATES v.8.2.0 program (Colwell, 2009), using 50 randomisations and sampling without replacement. As our data are based on presence–absence matrices, the ICE and Chao2 non-parametric estimators of the expected OTU richness were used.

Analysis of community structure

Co-occurrence patterns between OTUs were examined with Stone & Roberts (1990) *C*-score test, as implemented in ECOSIM 7.72 software (Gotelli & Entsminger, 2012). This metric accounts for the mean number of pairwise

comparisons where two OTUs (or, more generally, taxa) do not co-occur in the same site (or 'checkerboard unit') found across all pairwise taxa comparisons between sites (Stone & Roberts, 1990). The *C*-scores for observed data matrices were compared with the distribution of *C*-scores from a collection of 5000 simulated matrices generated by EcoSim under the following three null models (Gotelli, 2000; Gotelli & Entsminger, 2012):

1 Fixed–fixed model (FF model), which preserves the number of OTUs' occurrences across all sites (samples or habitats) and the number of OTUs at any given site from the observed matrix when generating each simulated matrix.

2 Fixed–equiprobable model (FE model), where OTUs' occurrence frequencies are again maintained while sites are assumed to have an equal probability of being colonised by any OTU.

3 Fixed–proportional model (FP model), in which site occurrences are not identical to those in the observed matrix but, on average, their rank order based on OTU richness in the simulated matrices matches that of the original data set. As in the previous models, the total number of site occurrences for each OTU is preserved.

A significantly higher observed *C*-score than the average from the simulated matrices is indicative of average pairwise species co-occurrence being less frequent than expected by chance and, therefore, a structured (i.e. non-random) community (Gotelli, 2000; Horner-Devine *et al.*, 2007; Gotelli & Entsminger, 2012). EcoSim also provides the standardised effect size (SES) for the observed *C*-scores, which scales the results in units of standard deviations and allows comparing the degree of co-occurrence across different data sets (Gotelli & Entsminger, 2012). A detailed description of these analyses is provided in Appendix S1.

To identify which OTUs were positively or negatively associated with each other, we constructed a co-occurrence matrix and calculated pairwise Pearson correlation coefficients. The corresponding *P*-values were adjusted by the sequential Bonferroni test, which corrects for multiple comparisons (Holm, 1979; Rice, 1989). These calculations were performed with R v.2.11.1 software (R Development Core Team, 2010), using the 'rcorr.adjust' function in the 'Hmisc' package.

Results

Occurrence and co-occurrence of bacteria and yeasts in floral nectar

Occurrence and co-occurrence patterns of culturable microorganisms in individual nectar samples are summarised in Table 1. The overall frequency of occurrence of bacteria and yeasts was 19.9% and 33.6% ($N = 1002$), respectively. Mycelial fungi were only recovered from a few samples (0.9%) and were excluded from analyses.

There was extensive variation among plant species in bacteria and yeast occurrence. While the proportion of nectar samples containing yeasts almost encompassed the whole 0–100% range, the proportion of nectar samples containing bacteria was below 80% in all the plant species analysed (Fig. 2). Bacteria were not recovered from floral nectar of 13 plant species (Table 1). Nectar yeasts were neither recovered from nine of these latter species. In the opposite extreme, bacteria occurred in a high proportion of nectar samples from some plants, such as *Iris xiphium* (71.4% of samples), *Lonicera implexa* (67.7%), *Antirrhinum graniticum* (66.7%), *Antirrhinum major* (66.7%), *Gladiolus illyricus* (60.7%) and *Convolvulus althaeoides* (57.1%).

The overall rate of bacteria–yeasts co-isolation from floral nectar was 15.9% ($N = 1002$), or 42.2% ($N = 377$) of all samples yielding some microorganisms in microbiological analyses. A highly significant statistical association was found between bacteria and yeasts recovery in cultures (Fisher's exact test, $P < 0.0001$). Furthermore, for the set of 44 plant species analysed, there was a significant direct association across species between the frequencies of occurrence of bacteria and yeasts ($R^2 = 0.45$, d.f. = 42, $P < 0.0001$; TIP analysis in Fig. 3). Such significant association was corroborated by PIC analysis ($R^2 = 0.45$, d.f. = 35, $P < 0.0001$; Fig. 3), thus confirming that it was not an artefact of phylogenetic correlations present in the data. When the relationship between bacteria and yeast occurrence was assessed separately by plant species, significant ($P < 0.05$ or better) associations were obtained in 17 cases (Table 1).

Observed and estimated OTU richness

A total of 28 bacterial and 20 yeast OTUs were identified at the 3% DNA dissimilarity cut-off ($OTUs_{0.03}$ hereafter) in the Mothur analysis of DNA sequence data. Ten additional bacterial OTUs and four yeast OTUs were identified when the dissimilarity cut-off was lowered to 1% ($OTUs_{0.01}$ hereafter), thus giving a total of 38 and 24 $OTUs_{0.01}$, respectively. Nevertheless, only $OTUs_{0.03}$ were considered in most subsequent analyses (see below), as this represents the threshold commonly used to distinguish microbial OTUs at the species level in studies on microbial ecology (e.g. Teixeira *et al.*, 2010; Martinson *et al.*, 2011) including previous reports of nectar microorganisms (Pozo *et al.*, 2011; Álvarez-Pérez *et al.*, 2012a; Fridman *et al.*, 2012; de Vega & Herrera, 2012).

When all nectar samples from all plant species were combined into a single analysis (drop-based approach),

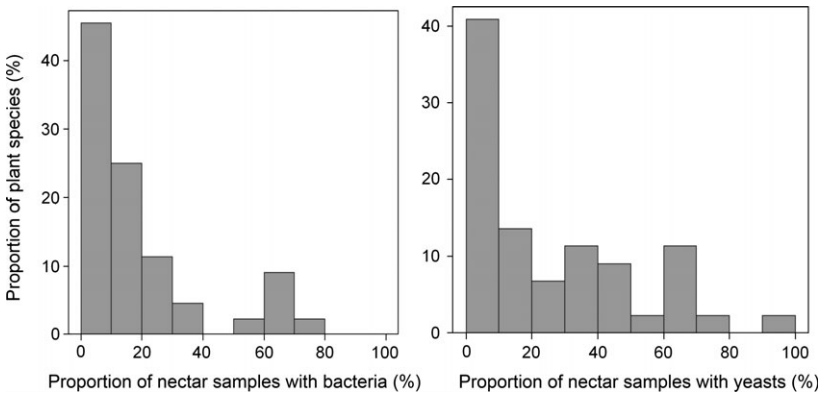

Fig. 2. Frequency distributions of the proportion of nectar samples from a given plant species that contained bacteria (left) or yeasts (right).

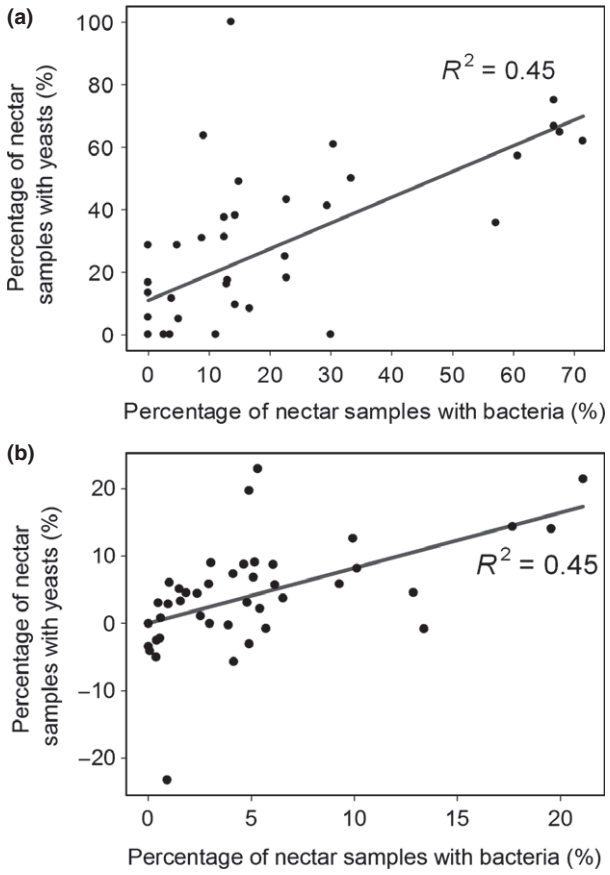

Fig. 3. Relationship between the percentages of occurrence of bacteria (*x*-axis) and yeasts (*y*-axis) in the floral nectar of the set of 44 plant species sampled in the present survey, as assessed by TIC (a) and PIC analyses (b). Points in the graphs represent individual plant species or phylogenetically independent contrasts, respectively. In both cases, the proportion of variance accounted for by the least squares fitted linear regression line is shown (R^2). In the PIC analysis, contrasts are positivised on the x variable (as recommended by Garland *et al.*, 1992).

the $OTU_{0.03}$ accumulation curves of bacterial and yeast communities were close to reaching a plateau for the number of nectar samples analysed ($N = 613$; Fig. 4). Nevertheless, additional OTUs were expected to appear in both cases with additional sampling effort and/or by lowering the DNA dissimilarity cut-off (see Table S2 and Fig. S1). For the yeast community, graphical representations of the ICE and Chao2 estimators of the expected $OTU_{0.03}$ richness approached the accumulation curve, which means a high recovery rate of culturable yeasts with our sampling ($\geq$ 79.4%, Table 2). In contrast, our sampling showed a poorer performance in recovering bacteria from nectar drops, as only between 42.4 and 49.9% of the expected number of culturable bacterial $OTUs_{0.03}$ were detected (Table 2). Furthermore, the ICE richness estimator for the bacterial community gradually drifted apart from the accumulation curve with increasing sampling effort. The Chao2 estimator showed a trend towards reaching a plateau at a higher $OTU_{0.03}$, thus corroborating that the observed OTU accumulation curve underestimated the culturable bacterial $OTU_{0.03}$ richness.

Rarefaction analyses using plant species as sampling units (plant species-based approach) yielded similar results. Bacterial and yeast $OTU_{0.03}$ accumulation curves and nonparametric estimators of OTU richness approached a plateau for the number of plant species sampled ($N = 31$, Fig. 4). As in the drop-based analysis, our sampling only detected between 44.2 and 49.2% of the total estimated culturable bacterial $OTU_{0.03}$ richness occurring in floral nectar in the regional plant community surveyed. The percentage of recovery of $OTUs_{0.03}$ was again higher for yeasts, especially when calculated in relation to the Chao2 nonparametric estimator (Table 2).

Microbial communities in individual nectar drops had very low OTU richness, as on average ± SE only 1.13 ± 0.03 yeast and 1.36 ± 0.07 bacterial $OTUs_{0.03}$ were recovered from each nectar drop sampled (0.28 ± 0.02 and 0.25 ± 0.02, respectively, if samples not yielding microbial growth are included, $N = 613$). The richest microbial communities occurred in the nectar of *C. althaeoides*

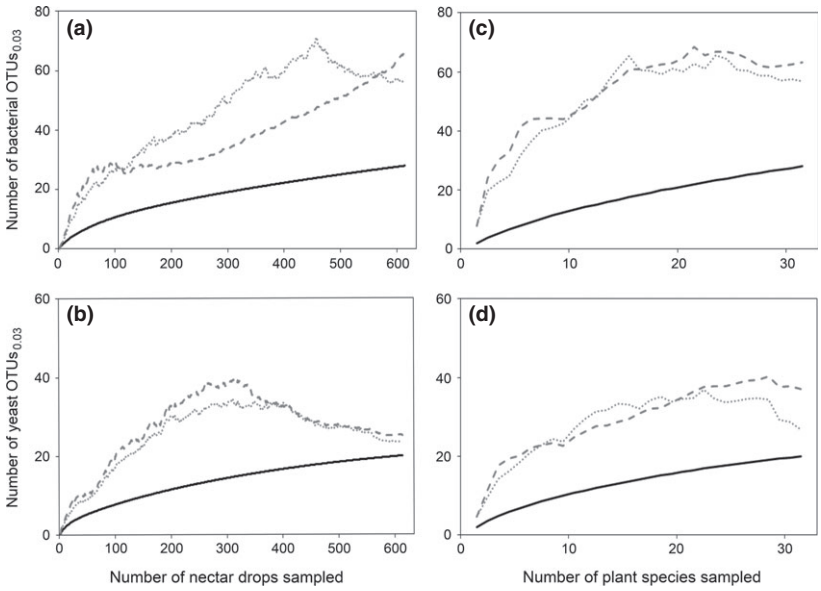

Fig. 4. Graphical representation of 'drop-based' (a, b) and 'plant species-based' (c, d) rarefaction curves (black continuous line) and nonparametric estimators of nectar microorganisms $OTU_{0.03}$ richness for our data set: ICE (grey dashed line) and Chao2 (grey dotted line). Panes a and c correspond to nectar bacteria, while b and d refer to nectar yeasts. For similar representations based on OTUs defined at the 1% DNA dissimilarity cut-off ($OTUs_{0.01}$), see Fig. S1.

Table 2. Observed and expected $OTU_{0.03}$ richness of nectar bacterial and yeast communities for the drop-based and plant-based rarefaction analyses

	Parameter	Bacterial community	Yeast community
Drop-based analysis	Observed number of $OTUs_{0.03}$	28	20
	ICE estimator	66.0	25.2
	Chao2 estimator	56.1	23.5
	$OTUs_{0.03}$ recovery*	42.4%/49.9%	79.4%/85.1%
Plant-based analysis	Observed number of $OTUs_{0.03}$	28	20
	ICE estimator	63.3	37.1
	Chao2 estimator	56.9	26.8
	$OTUs_{0.03}$ recovery	44.2%/49.2%	53.9%/74.6%

*Percentages of $OTUs_{0.03}$ recovery as calculated in relation to the ICE and Chao2 nonparametric estimators of expected richness, respectively.

(23 $OTUs_{0.03}$, 15 bacterial and eight fungal), *I. xiphium* (14 $OTUs_{0.03}$, eight bacterial and six fungal) and *G. illyricus* (13 $OTUs_{0.03}$, seven bacterial and six fungal).

Phylogenetic diversity of isolated nectar microorganisms

Comparison of rRNA gene sequences of bacterial isolates with reference sequences in databases showed a distribution among four major phyla: Actinobacteria (11.3% of isolates), Bacteroidetes (1.3%), Firmicutes (9.9%) and Proteobacteria (77.5%, belonging to alpha-, beta- and gammaproteobacteria) (Table 3). Yeasts and yeast-like fungi belonged to the phyla Ascomycota (81.9% of isolates) and Basidiomycota (18.1%) (Table 4). *Acinetobacter* and *Pseudomonas* were the commonest bacterial genera recovered (Table 3), although their overall incidence was low (occurring in 9.3% and 2.9% of nectar samples, and 41.9% and 22.6% of plant species, respectively). Among yeasts, dominant species were *Metschnikowia reukaufii* and *M. gruessii*, which occurred in 12.6% and 7.2% of nectar drops and 48.4% and 29.0% of plant species, respectively (Table 4). The rest of bacterial and yeast taxa occurred each in a small proportion of nectar samples and plant species, which led to rank-abundance curves with long right-hand tails typical of most microbial communities (Fig. 5).

Variation of nectar microbiota across plant species

A comparison at the phylum level of the nectar microbial communities associated with different plant species is shown in Fig. 6. There was extensive interspecific variation in the proportion of isolates belonging to each microbial phylum (Fig. 6). Plant species also differed in the proportion of isolates represented by each $OTU_{0.03}$ (data not shown), as exemplified by broad variation in the proportions of *M. reukaufii*, *M. gruessii* and *Acinetobacter* (Fig. 6).

Table 3. Taxonomic affiliation of bacterial $OTUs_{0.03}$ recovered in this work from floral nectar of Mediterranean plants

Taxon*,†	N‡	Host plants§
Actinobacteria		
Actinosynnemataceae	1	CA
Arthrobacter sp.	1	GI
Cellulomonas sp.	1	CA
Curtobacterium sp.	1	MC
Kocuria sp.	1	CA
Leifsonia sp.	1	EG
Microbacteriaceae	5	AC, CA, IX
Microbacterium sp.	4	BB, CA
Streptomyces sp.	2	GI, NP
Bacteroidetes		
Flavobacteriaceae	2	GI
Firmicutes		
Bacillus sp.	1	BB
Enterococcus sp.	1	IX
Lactococcus sp.	1	IX
Leuconostoc sp.	11	EG, NP
Staphylococcus sp.	1	CA
Proteobacteria		
Acetobacteraceae	1	CA
Acinetobacter¶	57	AC, CG, EB, EP, FL, GI, IX, LI, LS, LV, MC, RO, SA
Alcaligenaceae	2	CA
Enterobacteriaceae sp. A	11	AR, BB, CA, IX, MC, NP
Enterobacteriaceae sp. B	5	CA, GI, IX
Enterobacteriaceae sp. C	10	GI, IX, NP, ORG
Methylobacterium sp.	1	BB
Pseudomonas sp. A	15	CA, EG, FL, GI, IX, NP, OR
Pseudomonas sp. B	10	CA, EG
Rhizobiaceae	2	AG, CG
Rhizobium sp.	1	CA
Sphingomonadaceae	1	CA
Variovorax sp.	1	CA

*As determined by comparison with reference sequences stored in the GenBank and the Ribosomal Database Project (RDP) databases.
†To avoid confusion, OTUs representing different unnamed species belonging to the same genus of family were named as sp. A, B and C.
‡Number of isolates.
§Plant species in which the different bacterial taxa were found. See Table 1 for acronyms of plant names.
¶Including *Acinetobacter nectaris* and *A. boissieri* (see the Discussion section of this article and Álvarez-Pérez *et al.*, 2012b).

Table 4. Taxonomic affiliation of yeast and yeast-like $OTUs_{0.03}$ recovered in this work from floral nectar of Mediterranean plants

Taxon*,†	N‡	Host plants§
Ascomycota		
Aureobasidium pullulans	16	AC, AV, CA, EG, GI, IX, LA, LI, NP, PP
Candida magnoliae	1	IX
Metschnikowia gruessii	44	AC, EB, EP, GI, IX, LI, LS, RO, TF
Metschnikowia reukaufii	77	AC, CG, EB, EG, EP, GI, IX, LA, LI, LS, LV, MC, MO, NP, RO
Starmerella bombicola	2	AG, CA
Basidiomycota		
Cryptococcus laurentii	1	EG
Cryptococcus luteolus	1	CA
Cryptococcus macerans	1	NP
Cryptococcus sp. A	2	CA
Cryptococcus sp. B	5	BB, EG, VV
Cryptococcus sp. C	1	LI
Cryptococcus sp. D	4	CA, OR
Cryptococcus sp.E	2	AG, AV
Filobasidium sp.	4	GI, LI, MO, SA
Moniliella megachiliensis	1	GI
Rhodotorula nothofagi	2	CA, IX
Rhodotorula sp.	1	GI
Sporobolomyces roseus	2	AG, IX
Tremellales	2	AG, CA
Trimorphomyces sp.	2	CA

*As determined by comparison with reference sequences stored in the GenBank and the Ribosomal Database Project (RDP) databases.
†To avoid confusion, OTUs representing different unnamed species belonging to the same genus were named as species A–E.
‡Number of isolates.
§Plant species in which the different fungal taxa were found. See Table 1 for acronyms of plant names.

Community assembly

The observed and simulated *C*-scores for the four different data sets analysed (drop and plant based, each with either 3% or 1% DNA dissimilarity cut-off) are shown in Table 5. Observed *C*-scores were in most cases significantly higher than the expectations under the FF and FP null models, indicating lower average OTUs co-occurrence than expected by chance. The only exception was the FP model for the plant-based 3% cut-off data set ($P = 0.10$). In contrast, observed *C*-scores displayed a significantly lower value than simulations under the FE null model for all data sets, thus indicating fewer checkerboards and a higher co-occurrence frequency of OTUs than expected by chance (i.e. positive species co-occurrence).

Fifty-one significant associations, all of them of positive sign, were identified after calculating Pearson correlation coefficients for the $OTUs_{0.03}$ co-occurrence matrix. Most of these associations were poorly represented in the drop-based data set (presence in < 5% of samples yielding microorganisms in cultures). On the contrary, the following three bacterium–yeast pairs were not only significantly associated but also relatively frequent (Table 6): *Acinetobacter* + *M. gruessii*, *Acinetobacter* + *M. reukaufii* and *Leuconostoc* sp. + *M. reukaufii*.

Discussion

The traditional separation of microbiological research between bacteriologists and mycologists has often led to disconnected studies of bacteria and fungi, overlooking

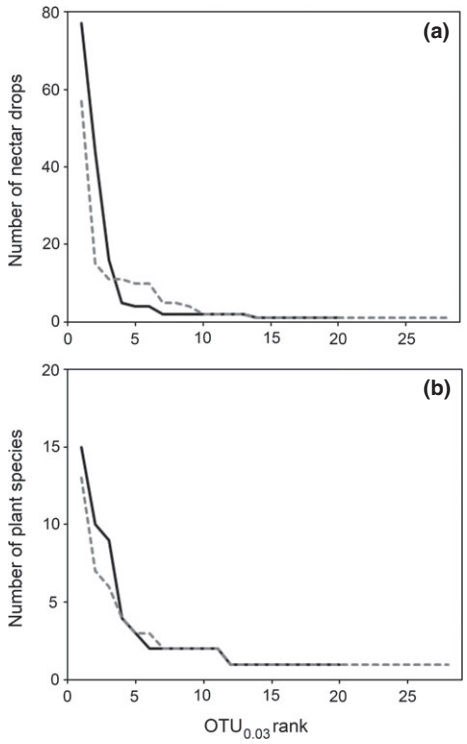

Fig. 5. Drop-based (a) and plant-based (b) rank-abundance curves for nectar bacterial (grey dotted line) and yeast communities (black solid line).

the fact that these microbial groups coexist in many environments and that bacterial–fungal interactions often have important consequences on the biology of the interacting partners (Wargo & Hogan, 2006; Frey-Klett *et al.*, 2011). Most previous studies on floral nectar microbiology have followed this canonical approach by focusing on either the yeast or the bacterial communities inhabiting nectar (e.g. Pozo *et al.*, 2011; Álvarez-Pérez *et al.*, 2012a; Fridman *et al.*, 2012). To get a more realistic perspective on the structure and diversity of nectar microbial communities, in this work we studied both the bacteria and microfungi found in the floral nectar of wild Mediterranean plant species. Nevertheless, as communities of nectar bacteria have been less extensively studied than those of yeasts and yeast-like microorganisms, we start this discussion by briefly addressing the occurrence patterns of the former in the plant community surveyed.

Bacteria in floral nectar

As observed for yeasts (Herrera *et al.*, 2009; Pozo *et al.*, 2011; and this study), the occurrence of culturable bacteria in the floral nectar of southern Spanish plants shows extensive interspecies variation, with some plants showing high frequencies of occurrence and others having virtually bacteria-free nectar. This result agrees also with patterns in frequency of occurrence of yeasts found in other biogeographical regions (Sandhu & Waraich, 1985; de Vega *et al.*, 2009). Overall frequency of occurrence of bacteria in nectar samples recorded in this work (19.9%) is noticeably lower than that found in floral nectar from South African plants, where bacteria were recovered from 53.5% of samples (Álvarez-Pérez *et al.*, 2012a). Differences between the South African and Spanish plant communities in some relevant aspects (e.g. species composition, main pollinators and phenology) might account for the lower occurrence of bacteria observed in the present study. As there are no available data on the prevalence of nectar bacteria in other plant communities, we cannot place our results in a more general context, but broad-scale geographical variation in bacterial incidence in floral nectar, if confirmed by future studies, prompts for biogeographical and ecological interpretations.

Species richness and composition of nectar communities

Detailed analyses of the species composition of nectar yeast communities associated with a wide variety of plants are available (Sandhu & Waraich, 1985; Brysch-Herzberg, 2004; Herrera *et al.*, 2010; Pozo *et al.*, 2011, 2012; Belisle *et al.*, 2012; de Vega & Herrera, 2012). Similarly, the composition of bacterial communities associated with floral nectar of some communities of wild (Álvarez-Pérez *et al.*, 2012a) and cultivated plants (Fridman *et al.*, 2012) has been recently studied. However, no effort has been made so far to compare nectar bacterial and yeast communities in terms of species richness. Our results showed that, in general terms, the culturable nectar-dwelling bacterial and yeast communities were similar in having low OTU richnesses, yet nonparametric estimators suggested that while our sampling procedure was suitable for recovering most yeast OTUs, it underestimated bacterial diversity. Further rare OTUs would appear if more nectar drops or if additional plant species were sampled. This expectation is consistent with the strongly right-skewed rank-abundance distributions of microbial OTUs found in this work, characterised by a few abundant and many rare OTUs, as generally observed in microbial communities (Hughes *et al.*, 2001; Fuhrman, 2009).

The present study confirmed *M. reukauffi* and *M. gruessii* as the main fungal inhabitants of floral nectar and identified *Acinetobacter* as the main nectar-dwelling bacteria in wild Mediterranean plants. This preponderance of *Acinetobacter* in floral nectar of wild plants from southern

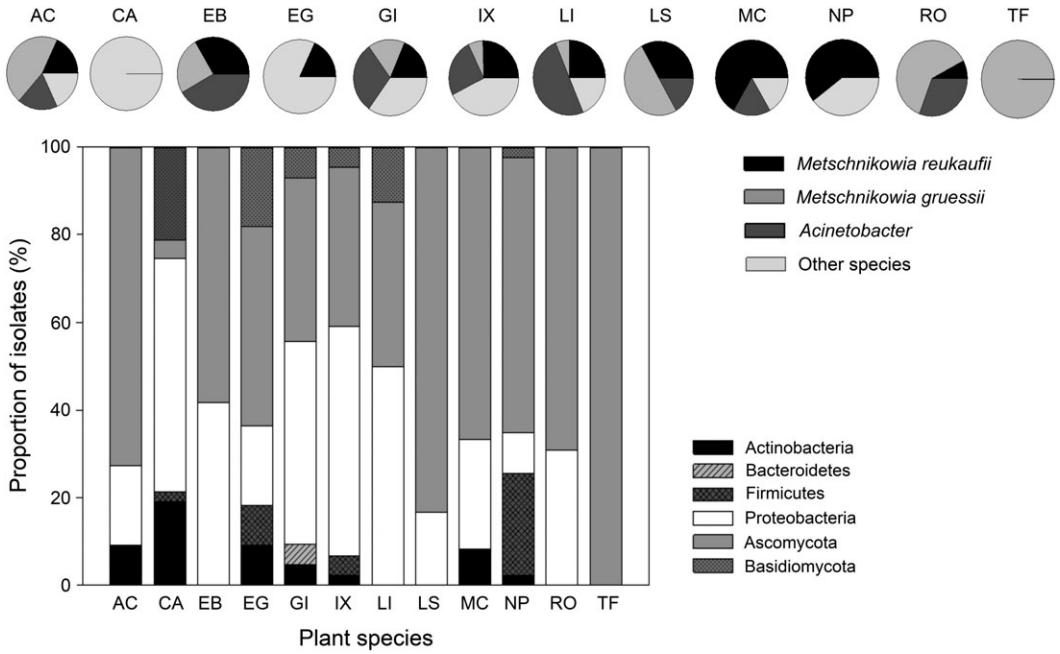

Fig. 6. Phylum level relative abundance profiles using 16S rRNA or D1/D2 26S rRNA gene sequence classifications for bacteria and fungi, respectively. Columns reflect the percentage of isolates assigned to each phylum using the Ribosomal Database Project (RDP) classifier and Basic Local Alignment Search Tool (BLAST) searches. Pie charts above columns represent, for each plant species (symbols as in Table 1), the proportion of isolates belonging to *Acinetobacter*, *Metschnikowia reukaufii*, *M. gruessii* or other species. Only those plant species from which $\geq$ 10 microbial isolates were recovered are shown.

Table 5. Summary of results of the analyses of microbial OTUs co-occurrence in floral nectar of Mediterranean plants

			Simulated *C*-scores[†]			
Data set	Null model*	Observed *C*-score	Mean	Variance	SES[‡]	*P*-value[§]
Drop-based, 3% cut-off[¶]	FF	31.04	28.20	0.09	9.26	< 0.0001
	FE	31.04	36.09	0.27	−9.70	< 0.0001*
	FP	31.04	23.52	0.67	9.19	< 0.0001
Drop-based, 1% cut-off	FF	20.05	18.36	0.04	8.27	< 0.0001
	FE	20.05	22.72	0.08	−9.34	< 0.0001*
	FP	20.05	15.72	0.24	8.87	< 0.0001
Plant-based, 3% cut-off	FF	3.24	2.70	< 0.01	5.68	< 0.0001
	FE	3.24	4.37	0.01	−10.83	< 0.0001*
	FP	3.24	2.98	0.05	1.22	NS
Plant-based, 1% cut-off	FF	2.84	2.37	< 0.01	6.04	< 0.0001
	FE	2.84	3.61	< 0.01	−12.22	< 0.0001*
	FP	2.84	2.55	0.03	1.81	0.03

*FF: fixed–fixed; FE: fixed–equiprobable; FP: fixed–proportional. For further details on these null models, see Materials and Methods.
[†]Values obtained from 5000 randomised matrices.
[‡]Standardised effect size.
[§]*P*-values refer to 'observed *C*-score $\geq$ expected *C*-score' comparisons, except those denoted by an asterisk, which are *P*-values for the opposite comparisons. NS: not significant ($P > 0.05$).
[¶]DNA dissimilarity cut-off.

Spain agrees with observations on nectar bacterial communities of cultivated plants in Israel, where species from this genus occurred in 49–90% of samples studied (Fridman *et al.*, 2012). In contrast, *Acinetobacter* seems to be rare in nectar communities of wild South African plants, where other Proteobacteria are more prevalent (e.g. *Pseudomonas*; Álvarez-Pérez *et al.*, 2012a; and unpublished data). Remarkably, the *Acinetobater* isolates studied in this

Table 6. Significant pairwise associations between nectar microorganisms

$OTUs_{0.03}$ pair*	Pearson correlation coefficient	*P*-value†	Relative frequency (%)‡	Plant hosts§
Acinetobacter + *Metschnikowia gruessii*	0.61	0.0047	10.7	AC, EB, EP, GI, IX, LI, LS, RO
Acinetobacter + *Metschnikowia reukaufii*	0.71	< 0.0001	16.9	AC, EB, EP, GI, IX, LI, LS, LV, MC, RO
Leuconostoc sp. + *Metschnikowia reukaufii*	0.69	< 0.0001	5.6	NP

*Only those pairs of nectar microorganisms significantly correlated and with a relative frequency $\geq$ 5% are shown.
†Adjusted *P*-values, as calculated after correcting for multiple comparisons by the sequential Bonferroni test.
‡Percentage of nectar samples in relation to those yielding microbial growth in plate cultures (N = 178).
§Plant species where the association between microorganisms was found. For acronyms of plant names, see Table 1.

work grouped into a single OTU defined on the basis of a 3% dissimilarity cut-off in the 16S rRNA gene, but into two different OTUs when this threshold was lowered to 1% (data not shown). These two $OTUs_{0.01}$ also differed in other genetic and phenotypic traits, leading to the recent description of two novel species within the genus *Acinetobacter*, which have been named as *A. nectaris* and *A. boissieri* (Álvarez-Pérez *et al.*, 2012b).

Structure of nectar-inhabiting microbial communities

Nectarivorous bacteria and yeasts were found coexisting more often than would be expected by chance, as denoted by highly significant positive association across plant species and across samples from the same species. Both TIC and PIC analyses showed a significant positive association between the frequencies of single occurrence of nectar-living bacteria and yeasts, which was independent from phylogenetic relationships among the plant species surveyed.

Further supporting the nonrandom assembly of nectar microbiota are the results of analyses of co-occurrence between microbial OTUs in a representative subset of nectar samples using the *C*-score test. Almost without exception, we obtained *C*-scores significantly higher than those resulting from computer simulations, indicating that OTUs co-occurrences were less frequent than expected by chance. On the contrary, observed *C*-scores were in all cases lower than simulations under the FE null model, therefore suggesting positive species co-occurrences. These seemingly contradictory results can be explained by taking into account some methodological considerations, which are discussed in detail in Appendix S1. Briefly, the FE model assumes that all the sites are of similar size and quality (Gotelli, 2000). Clearly, this is not the case of floral nectar as broad inter- and intraspecific variation in different nectar traits has been described (see, e.g., Herrera *et al.*, 2006; Canto *et al.*, 2011). Therefore, if the FF or FP null models are regarded as the best suited for the analysis of OTUs incidence matrices of nectar microbial communities, our results support the hypothesis of lower co-occurrence among microbial OTUs than expected by chance and thus nonrandom assembly of the nectar microbiota.

Possibly the most remarkable signature of nectar community structure found in this study were the 51 significant positive associations between bacteria and yeasts, as they denote nonrandom co-assembly of two disparate groups of microorganisms that usually tend to be analysed separately. Most of such associations occurred in < 5% of nectar drops yielding culturable microorganisms, which points to a limited ecological relevance. Nevertheless, three bacterium–yeast associations involving yeasts from the *Metschnikowia* clade were fairly frequent. The two most prevalent of these positive associations, namely *Acinetobacter* + *M. gruessii* and *Acinetobacter* + *M. reukaufii*, were found in different plant hosts, while the third one, whose partners were *Leuconostoc* sp. and *M. reukaufii*, was exclusively discovered in nectar of the winter-blooming plant *Narcissus papyraceus*.

Questions for future studies

Nonrandom co-occurrence patterns between taxa may indicate that deterministic processes are important in structuring communities but, by themselves, do not identify the causal mechanisms accounting for such patterning (Horner-Devine *et al.*, 2007). Low co-occurrence is usually interpreted as evidence of divergence in habitat preference, differences in growth constraints between taxa, and/or negative interactions (Gotelli & McCabe, 2002; Horner-Devine *et al.*, 2007; Pan & May, 2009; Eiler *et al.*, 2011; Parnell & Streelman, 2011). Oppositely, positive species co-occurrences are usually attributed to different species having similar environmental requirements and not showing competitive exclusion, and/or positive species interactions (Horner-Devine *et al.*, 2007; Pan & May, 2009; Eiler *et al.*, 2011; Parnell & Streelman, 2011). Furthermore, in the case of nectar microorganisms, positive co-occurrence might be the result of flower colonisation via the same dispersal vectors, mainly flower-visiting insects (Pozo *et al.*, 2012). Finally, historical events such as priority effects (i.e. interactions between species depending on

their order of arrival to a niche) could also shape some microbial communities (Peay *et al.*, 2012). Given the multitude of possible pairwise combinations between the microbial OTUs identified in this work, different combinations of the aforementioned mechanisms could result in the observed patterns on nonrandom assembly of nectar communities. Further exploring associations and co-occurrence patterns between nectar microorganisms could help to disclose biotic interactions, habitat affinities or shared physiologies, and might reveal hitherto unexplored relationships linking fungal and bacterial communities with nectar features and the functionality of plant–pollinator interactions.

Although we are conscious that the diversity of the culturable fraction of most microbial communities may underestimate actual diversity (Amann *et al.*, 1995; Fry, 2000; but see Donachie *et al.*, 2007), in this work we focused on the culturable bacterial and yeast communities inhabiting floral nectar. Owing to the large number of nectar samples included in our survey of nectar microorganisms, the scarce amount of floral nectar produced by most plant species sampled (usually < 2 μL) and the drop-based approach used for data analyses – which precludes mixing nectar samples from different plant individuals – an adequate study of the unculturable diversity of nectar microorganisms would have been methodologically challenging. This kind of study would require selecting a few model plant species producing abundant floral nectar, which, at least in Mediterranean ecosystems, clearly would bias the sampling of the plant community towards a few species.

Finally, it is becoming clear that microbial species can exhibit biogeographical patterns (Martiny *et al.*, 2006), with some taxa being exclusively found or particularly frequent in some places or habitats but (practically) absent from others. Although still very limited, there is some evidence that the distribution of certain nectar microorganisms might follow a similar pattern (see, e.g., our comments above on *Acinetobacter*). As different sets of microorganisms usually differ on the array of interspecies interactions they display, it would be worth investigating whether nonrandom assembly of the nectar microbiota, as observed in this work for Mediterranean insect-pollinated plants, also holds true for other plant communities of different floristic regions.

Acknowledgements

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References

Álvarez-Pérez S, Herrera CM & de Vega C (2012a) Zooming-in on floral nectar: a first exploration of nectar-associated bacteria in wild plant communities. *FEMS Microbiol Ecol* **80**: 591–602.

Álvarez-Pérez S, Lievens B, Jacquemyn H & Herrera CM (2012b) *Acinetobacter nectaris* sp. nov. and *Acinetobacter boissieri* sp. nov., two novel bacterial species isolated from floral nectar of wild Mediterranean insect-pollinated plants. *Int J Syst Evol Microbiol*, doi: 10.1099/ijs.0.043489-0.

Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143–169.

Archie EA & Theis KR (2011) Animal behaviour meets microbial ecology. *Anim Behav* **82**: 425–436.

Belisle M, Peay KG & Fukami T (2012) Flowers as islands: spatial distribution of nectar-inhabiting microfungi among plants of *Mimulus aurantiacus*, a hummingbird-pollinated shrub. *Microb Ecol* **63**: 711–718.

Blanca G, Cabezudo B, Cueto M, Fernández-López C & Morales-Torres C (2009) *Flora Vascular de Andalucía Oriental*, **4** vols. Consejería de Medio Ambiente, Junta de Andalucía, Sevilla.

Brandenburg A, Dell'Olivo A, Bshary R & Kuhlemeier C (2009) The sweetest thing. Advances in nectar research. *Curr Opin Plant Biol* **12**: 486–490.

Brysch-Herzberg M (2004) Ecology of yeasts in plant-bumblebee mutualism in Central Europe. *FEMS Microbiol Ecol* **50**: 87–100.

Canto A, Herrera CM, García IM, Pérez R & Vaz M (2011) Intraplant variation in nectar traits in *Helleborus foetidus* (Ranunculaceae) as related to floral phase, environmental conditions and pollinator exposure. *Flora* **206**: 668–675.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* **17**: 540–552.

Chanbusarakum L & Ullman D (2008) Characterization of bacterial symbionts in *Frankliniella occidentalis* (Pergande), Western flower thrips. *J Invertebr Pathol* **99**: 318–325.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG & Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31**: 3497–3500.

Colwell RK (2009) *EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples. Version 8.2*. User's Guide and application published at: http://purl.oclc.org/estimates

Donachie SP, Foster JS & Brown MV (2007) Culture clash: challenging the dogma of microbial diversity. *ISME J* **1**: 97–99.

Ehlers BK & Olesen JM (1997) The fruit-wasp route to toxic nectar in *Epipactis* orchids? *Flora* **192**: 223–229.

Eiler A, Hayakawa DH & Rappé MS (2011) Non-random assembly of bacterioplankton communities in the subtropical North Pacific Ocean. *Front Microbiol* **2**: 140.

Eisikowitch D, Lachance M-A, Kevan PG, Willis S & Collins-Thompson DL (1990) The effect of the natural assemblage of microorganisms and selected strains of the yeast *Metschnikowia reukaufii* in controlling the germination of pollen of the common milkweed *Asclepias syriaca*. *Can J Bot* **68**: 1163–1165.

Fell JW, Boekhout T, Fonseca A, Scorzetti G & Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**: 1351–1371.

Felsenstein J (1985) Phylogenies and the comparative method. *Am Nat* **125**: 1–15.

Fierer N & Lennon JT (2011) The generation and maintenance of diversity in microbial communities. *Am J Bot* **98**: 439–448.

Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M & Sarniguet A (2011) Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiol Mol Biol Rev* **75**: 583–609.

Fridman S, Izhaki I, Gerchman Y & Halpern M (2012) Bacterial communities in floral nectar. *Environ Microbiol Rep* **4**: 97–104.

Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL & Martinez-Romero E (2011) Microbially mediated plant functional traits. *Annu Rev Ecol Evol Syst* **42**: 23–46.

Fry J (2000) Bacterial diversity and 'unculturables'. *Microbiol Today* **27**: 186–188.

Fuhrman JA (2009) Microbial community structure and its functional implications. *Nature* **459**: 193–199.

Garland T Jr & Díaz-Uriarte R (1999) Polytomies and phylogenetically independent contrasts: examination of the bounded degrees of freedom approach. *Syst Biol* **48**: 547–558.

Garland T Jr, Harvey PH & Ives AR (1992) Procedures for the analysis of comparative data using phylogenetically independent contrasts. *Syst Biol* **41**: 18–32.

Gilliam M, Moffett JO & Kauffeld NK (1983) Examination of floral nectar of *Citrus*, cotton, and Arizona desert plants for microbes. *Apidologie* **14**: 299–302.

Gotelli NJ (2000) Null model analysis of species co-occurrence patterns. *Ecology* **81**: 2606–2621.

Gotelli NJ & Entsminger GL(2012) *EcoSim: Null Models Software for Ecology, v. 7.0*. Acquired Intelligence Inc. & Kesey-Bear. Jericho, VT. Available at: http://garyentsminger.com/ecosim/index.htm

Gotelli NJ & McCabe DJ (2002) Species co-occurrence: a meta-analysis of J. M. Diamond's assembly rules model. *Ecology* **83**: 2091–2096.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* **41**: 95–98.

Heil M (2011) Nectar: generation, regulation and ecological functions. *Trends Plant Sci* **16**: 191–200.

Herrera CM, Pérez R & Alonso C (2006) Extreme intraplant variation in nectar sugar composition in an insect-pollinated perennial herb. *Am J Bot* **93**: 575–581.

Herrera CM, García IM & Pérez R (2008) Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants. *Ecology* **89**: 2369–2376.

Herrera CM, de Vega C, Canto A & Pozo MI (2009) Yeasts in floral nectar: a quantitative survey. *Ann Bot* **103**: 1415–1423.

Herrera CM, Canto A, Pozo MI & Bazaga P (2010) Inhospitable sweetness: nectar filtering of pollinator-borne inocula leads to impoverished, phylogenetically clustered yeast communities. *Proc R Soc B Biol Sci* **277**: 747–754.

Holm S (1979) A simple sequentially rejective multiple test procedure. *Scand J Stat* **6**: 65–70.

Horner-Devine MC & Bohannan BJM (2006) Phylogenetic clustering and overdispersion in bacterial communities. *Ecology* **87**: S100–S108.

Horner-Devine MC, Silver JM, Leibold MA *et al.* (2007) A comparison of taxon co-occurrence patterns for macro- and microorganisms. *Ecology* **88**: 1345–1353.

Hughes JB, Hellmann JJ, Ricketts TH & Bohannan BJM (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl Environ Microbiol* **67**: 4399–4406.

Konopka A (2009) What is microbial community ecology? *ISME J* **3**: 1223–1230.

Kurtzman CP & Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* **73**: 331–371.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techn-iques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. John Wiley and Sons, New York, NY.

Leveau JHJ & Preston GM (2008) Bacterial mycophagy: definition and diagnosis of a unique bacterial–fungal interaction. *New Phytol* **177**: 859–876.

Maddison WP & Maddison DR (2011) Mesquite: a modular system for evolutionary analysis, version 2.75. Available at: http://mesquiteproject.org

Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S & Moran NA (2011) A simple and distinctive microbiota associated with honey bees and bumble bees. *Mol Ecol* **20**: 619–628.

Martiny JBH, Bohannan BJM, Brown JH *et al.* (2006) Microbial biogeography: putting microorganisms on the map. *Nat Rev Microbiol* **4**: 102–112.

Midford PE, Garland T & Maddison W (2010) PDAP:PDTREE package for Mesquite, version 1.15. Available at: http://mesquiteproject.org/pdap_mesquite/

Nemergut DR, Costello EK, Hamady M *et al.* (2011) Global patterns in the biogeography of bacterial taxa. *Environ Microbiol* **13**: 135–144.

Nicolson SW & Thornburg RW (2007) Nectar chemistry. *Nectaries and Nectar* (Nicolson SW, Nepi M & Pacini E, eds), pp. 215–263. Springer-Verlag, Dordrecht.

Pan JJ & May G (2009) Fungal-fungal associations affect the assembly of endophyte communities in maize (*Zea mays*). *Microb Ecol* **58**: 668–678.

Parnell NF & Streelman JT (2011) The macroecology of rapid evolutionary radiation. *Proc R Soc B Biol Sci* **278**: 2486–2494.

Peay KG, Belisle M & Fukami T (2012) Phylogenetic relatedness predicts priority effects in nectar yeast communities. *Proc R Soc B Biol Sci* **279**: 749–758.

Pozo MI, Herrera CM & Bazaga P (2011) Species richness of yeast communities in floral nectar of southern Spanish plants. *Microb Ecol* **61**: 82–91.

Pozo MI, Lachance M-A & Herrera CM (2012) Nectar yeasts of two southern Spanish plants: the roles of immigration and physiological traits in community assembly. *FEMS Microbiol Ecol* **80**: 281–293.

Purvis A & Garland T Jr (1993) Polytomies in comparative analyses of continuous characters. *Syst Biol* **42**: 569–575.

R Development Core Team (2010) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org

Reysenbach AL, Longnecker K & Kirshtein J (2000) Novel bacterial and archaeal lineages from an *in situ* growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. *Appl Environ Microbiol* **66**: 3798–3806.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution* **43**: 223–225.

Sandhu DK & Waraich MK (1985) Yeasts associated with pollinating bees and flower nectar. *Microb Ecol* **11**: 51–58.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**: 7537–7541.

Stone L & Roberts A (1990) The checkerboard score and species distributions. *Oecologia* **85**: 74–79.

Teixeira LC, Peixoto RS, Cury JC, Sul WJ, Pellizari VH, Tiedje J & Rosado AS (2010) Bacterial diversity in rhizosphere soil from Antarctic vascular plants of Admiralty Bay, maritime Antarctica. *ISME J* **4**: 989–1001.

Torsvik V, Øvreås L & Thingstad TF (2002) Prokaryotic diversity – magnitude, dynamics, and controlling factors. *Science* **296**: 1064–1066.

Valdés B, Talavera S & Fernández-Galiano E (1987) *Flora vascular de Andalucía occidental*, **3** vols Ketres Editora S.A., Barcelona.

de Vega C & Herrera CM (2012) Relationships among nectar-dwelling yeasts, flowers and ants: patterns and incidence on nectar traits. *Oikos*, doi: 10.1111/j.1600-0706.2012.20295.x

de Vega C, Herrera CM & Johnson SD (2009) Yeasts in floral nectar of some South African plants: quantification and associations with pollinator type and sugar concentration. *S Afr J Bot* **75**: 798–806.

Wäckers FL, Romeis J & van Rijn P (2007) Nectar and pollen feeding by insect herbivores and implications for multitrophic interactions. *Annu Rev Entomol* **52**: 301–323.

Wargo MJ & Hogan DA (2006) Fungal–bacterial interactions: a mixed bag of mingling microbes. *Curr Opin Microbiol* **9**: 359–364.

Webb CO & Donoghue MJ (2005) Phylomatic: tree assembly for applied phylogenetics. *Mol Ecol Notes* **5**: 181–183.

Webb CO, Ackerly DD & Kembel SW (2008) Phylocom: software for the analysis of phylogenetic community structure and trait evolution. *Bioinformatics* **24**: 2098–2100.

Whitman WB, Coleman DC & Wiebe WJ (1998) Prokaryotes: the unseen majority. *P Natl Acad Sci USA* **95**: 6578–6583.

Wiens F, Zitzmann A, Lachance M-A, Yegles M, Pragst F, Wurst FM, von olst D, Guan SL & Spanagel R (2008) Chronic intake of fermented floral nectar by wild treeshrews. *P Natl Acad Sci USA* **105**: 10426–10431.

Wikström N, Savolainen V & Chase MW (2001) Evolution of the angiosperms: calibrating the family tree. *Proc R Soc B Biol Sci* **268**: 2211–2220.

Zilber-Rosenberg I & Rosenberg E (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol Rev* **32**: 723–735.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Graphical representation of 'drop-based' (a, b) and 'plant species-based' (c, d) rarefaction curves (black line) and nonparametric estimators of nectar microorganisms $OTU_{0.01}$ richness for our dataset: ICE (gray dashed line) and Chao2 (gray dotted line).

Table S1. Family affiliation of the plant species examined in this work for presence of nectar microorganisms.

Table S2. Observed and expected $OTU_{0.01}$ richness of nectar bacterial and yeast communities for the drop-based and plant-based analyses.

Appendix S1. Detailed information on the *C*-score test.