

# Zooming-in on floral nectar: a first exploration of nectar-associated bacteria in wild plant communities

Sergio Álvarez-Pérez, Carlos M. Herrera & Clara de Vega

Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Sevilla, Spain

**Correspondence:** Sergio Álvarez-Pérez, Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Américo Vespucio, E-41092 Sevilla, Spain. Tel.: +34 954466700; fax: +34 954621125; e-mail: sealperez@ebd.csic.es

Received 30 October 2011; revised 31 January 2012; accepted 31 January 2012.  
Final version published online 8 March 2012.

DOI: 10.1111/j.1574-6941.2012.01329.x

Editor: Angela Sessitsch

## Keywords

bacteria; floral nectar; microbial communities; pollination.

## Abstract

Floral nectar of some animal-pollinated plants usually harbours highly adapted yeast communities which can profoundly alter nectar characteristics and, therefore, potentially have significant impacts on plant reproduction through their effects on insect foraging behaviour. Bacteria have also been occasionally observed in floral nectar, but their prevalence, phylogenetic diversity and ecological role within plant–pollinator–yeast systems remains unclear. Here we present the first reported survey of bacteria in floral nectar from a natural plant community. Culturable bacteria occurring in a total of 71 nectar samples collected from 27 South African plant species were isolated and identified by 16S rRNA gene sequencing. Rarefaction-based analyses were used to assess operational taxonomic units (OTUs) richness at the plant community level using nectar drops as sampling units. Our results showed that bacteria are common inhabitants of floral nectar of South African plants (53.5% of samples yielded growth), and their communities are characterized by low species richness (18 OTUs at a 16S rRNA gene sequence dissimilarity cut-off of 3%) and moderate phylogenetic diversity, with most isolates belonging to the *Gamma-proteobacteria*. Furthermore, isolates showed osmotolerance, catalase activity and the ability to grow under microaerobiosis, three traits that might help bacteria to overcome important factors limiting their survival and/or growth in nectar.

## Introduction

Plants provide extraordinarily diverse habitats for microorganisms (Andrews & Harris, 2000). Plant-associated habitats, such as roots, leaves, flowers, fruits or decaying tissues, differ in their local availability of nutrients and physicochemical conditions, thus filtering the range of potential microbial inhabitants (Andrews & Harris, 2000; Herrera *et al.*, 2010). From a microbiological perspective, only roots have been extensively investigated, especially on topics related to rhizosphere microbial communities and mycorrhizal or legume–*Rhizobium* symbioses (Long, 1996; Andrews & Harris, 2000; Kent & Triplett, 2002; Smith & Read, 2008). However, other plant microhabitats remain virtually unexplored. This latter is the case for floral nectar.

Historically, floral nectar has been regarded merely as a sweet aqueous secretion, containing sugars and amino acids, offered by flowering plants to attract pollinators

(Nicolson & Thornburg, 2007). Nevertheless, pollinators act not only as pollen vectors while visiting plants, but at the same time they can move microorganisms from flower to flower (Sandhu & Waraich, 1985; Brysch-Herzberg, 2004; Herrera *et al.*, 2009). As a rich source of nutrients, nectar could harbour a microbiota that may consume sugar actively and produce a range of metabolites, entailing a decrease in its attractiveness and energetic value from the viewpoint of pollinators. Thus, it has been postulated that plants should have evolved mechanisms to maintain nectar free of microorganisms (Adler, 2000; Carter & Thornburg, 2004). In this respect, in recent years several classes of antimicrobial proteins and secondary compounds putatively protecting nectar from microbial invasion have been isolated (Carter *et al.*, 2007; Park & Thornburg, 2009; Heil, 2011). For example, it has been hypothesized that reactive oxygen species, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), keep nectar palatable for visiting pollinators by limiting microbial growth,

thereby preventing toxin build up and reducing the breakdown of sugars and other nectar components by microbial metabolism (Carter & Thornburg, 2004; Carter *et al.*, 2007). Despite these assumptions, several recent studies have revealed that floral nectar of animal-pollinated plants from different continents and a variety of disparate habitat types can harbour highly adapted yeast communities (Brysch-Herzberg, 2004; Herrera *et al.*, 2008, 2009, 2010; de Vega *et al.*, 2009; Pozo *et al.*, 2011; de Vega & Herrera, 2012). Furthermore, it has been demonstrated that nectarivorous yeasts can profoundly alter both the sugar and amino acid composition and the overall energetic content of nectar as a consequence of their metabolic activity (Canto *et al.*, 2007, 2008; Herrera *et al.*, 2008; de Vega *et al.*, 2009; de Vega & Herrera, 2012; Peay *et al.*, 2012) and warm the flowers of some winter-blooming plants (Herrera & Pozo, 2010). Therefore, these eukaryotic microorganisms could have significant effects on plant reproduction through their effects on insect foraging behaviour (Herrera *et al.*, 2010).

The handful of studies that have addressed so far the topic of nectar microbiota mostly deal with yeasts (but see Junker *et al.*, 2011; which studies the microbiota of floral surfaces). Bacteria have also been occasionally observed in the nectar of some plants (Gilliam *et al.*, 1983; Ehlers & Olesen, 1997), but their prevalence and phylogenetic diversity have not been assessed to date. Furthermore, the ecological and functional role of these microorganisms in plant–pollinator–yeast systems remains unclear. Bacteria can degrade sugars, transform them into compounds which are difficult to assimilate by other microorganisms, produce alcohols, and release a wide array of secondary metabolites which are toxic to yeasts and/or insects (Latour & Lemanceau, 1997; Ström *et al.*, 2002; Barton, 2005; Bode, 2009). Hence, their potential impact on plant pollination cannot be ruled out.

Here we present the first reported survey of bacteria in floral nectar from a natural plant community. Our main objectives were to assess the prevalence, species richness and phylogenetic diversity of bacteria in a set of nectar samples from wild-growing South African plants. In this regard, the present report complements our previous work on nectar yeasts associated with the same South African plant community (de Vega *et al.*, 2009). Additionally, we determined three physiological characteristics of bacterial isolates that might be relevant for their survival in floral nectar, namely osmotolerance, catalase activity and the ability to grow under microaerobiosis. These traits might help bacteria to overcome, respectively, high sucrose levels, toxic hydrogen peroxide and possible oxygen limitation (e.g. derived from microbial metabolism) occurring in nectar. These analyses allowed the

identification of future research directions in nectar microbiology.

## Materials and methods

### Samples and microbiological analysis

Seventy-one samples of floral nectar from 27 plant species belonging to 13 families were analysed. A complete list of species sampled and the family to which they belong is provided in Table 1. Floral nectar collection was carried out at several localities in the KwaZulu-Natal province of South Africa differing in ecological characteristics including elevation, soil and type of vegetation (for further information, see de Vega *et al.*, 2009). All plants belonging to the same species were collected on the same location and, in some cases, plants of different species co-occurred. Sampled individuals were at least 5 m apart.

**Table 1.** Isolation of bacteria from nectar samples of 27 South African plant species

Plant family*	Species	No. †	Bacteria frequency‡
Acanthaceae	<i>Adhatoda andromeda</i>	3	2
	<i>Ruellia cordata</i>	3	1
	<i>Thunbergia natalensis</i>	2	1
Amaryllidaceae	<i>Haemanthus humilis</i>	3	1
Caryophyllaceae	<i>Silene bellidioides</i>	3	2
Ericaceae	<i>Erica cerinthoides</i>	3	1
Fabaceae	<i>Eriosema distinctum</i>	1	1
Iridaceae	<i>Dierama luteo-albidum</i>	3	0
	<i>Freesia laxa</i>	2	2
	<i>Gladiolus appendiculatus</i>	1	0
	<i>Gladiolus longicollis</i>	3	1
	<i>Gladiolus parvulus</i>	3	2
	<i>Moraea unibracteata</i>	2	2
	<i>Watsonia lepida</i>	1	0
	<i>Watsonia pillansii</i>	3	0
Lamiaceae	<i>Ajuga ophrydis</i>	3	2
	<i>Stachys aethiopica</i>	1	0
Orchidaceae	<i>Disa crassicornis</i>	2	2
Orobanchaceae	<i>Cycnium adonense</i>	4	4
	<i>Cycnium racemosum</i>	4	2
	<i>Graderia scabra</i>	1	1
Proteaceae	<i>Protea caffra</i>	4	4
	<i>Protea roupelliae</i>	3	0
Rubiaceae	<i>Burchellia bubalina</i>	3	2
Scrophulariaceae	<i>Glumicalyx goseloides</i>	6	2
Xanthorrhoeaceae	<i>Kniphofia caulescens</i>	1	1
	<i>Kniphofia</i> sp.	3	2
Total		71	38 (53.5%)

\*Familial classification follows that of Stevens (2011).

†Number of nectar samples analysed per plant species.

‡Number of nectar samples from which bacteria were isolated.

Collected branches, inflorescences or flowers already open and exposed to pollinators were carefully placed in plastic jars in a portable cooler until taken indoors, and then kept under refrigeration (4 °C) until further processing. Extractions of nectar from individual flowers, using sterile calibrated microcapillaries (Fisher Scientific, Madrid, Spain), were conducted within 24 h after field collection. Nectar standing crop varied widely depending on the plant species, ranging from approximately 1–3 µL per flower in *Adhatoda andromeda*, *Ajuga ophrydis*, *Cycnium adonense*, *Cycnium racemosum*, *Dierama luteoalbidum*, *Disa crassicornis*, *Eriosema distinctum*, *Freesia laxa*, *Gladiolus appendiculatus*, *Gladiolus parvulus*, *Glumicalyx goseloides*, *Graderia scabra*, *Haemanthus humilis*, *Moraea unibracteata*, *Ruellia cordata*, *Silene bellidioides* and *Stachys aethiopica*; 3–7 µL per flower in *Burchellia bubalina*, *Erica cerinthoides*, *Kniphofia* sp., *Thunbergia natalensis*, *Watsonia lepida* and *Watsonia pillansii*; to more than 30 µL per flower in *Gladiolus longicollis*, *Kniphofia caulescens*, *Protea caffra* and *Protea roupelliae*. Nectar sugar concentration was measured for some plant species with a low-volume hand refractometer (Bellingham & Stanley Ltd, Tunbridge Wells, UK), and exhibited extensive variation at both the inter- and intraspecific level. For example, the values of sugar concentration (in per cent of sucrose equivalents) for some of these species were as follows: *G. longicollis* (11–35%, mean 26.5%), *K. caulescens* (7–16%, mean 12%), *P. caffra* (2–12%, mean 7%), *P. roupelliae* (2–10%, mean 6%), *G. appendiculatus* (19–31%, mean 25%), *W. lepida* (10–29%, mean 21%) and *W. pillansii* (8.5–25%, mean 16%).

Nectar samples were immediately diluted in 500 µL of ultrapure deionized water (Purite Select; Purite Ltd, Thame, UK), and stored at 4 °C until processed. This procedure has proven to be similar to other methods (e.g. diluting in 0.85–1% NaCl solutions) in maintaining nectar microbiota in optimal conditions. Twenty-five microlitres of nectar dilutions was streaked on trypticase soy agar (TSA; Panreac, Castellar del Vallès, Spain). Cultures were incubated at room temperature (c. 25 °C) for 7 days. A colony of each phenotypically distinct type was picked and separately subcultivated on TSA to obtain pure cultures. All isolates were stored at –20 °C in Luria–Bertani (LB) broth (Difco, Sparks, MD) containing 25% glycerol (Sigma-Aldrich, Madrid, Spain).

### Phenotypic characterization of bacterial isolates

Bacterial isolates were further characterized by assessing their reaction to hydrogen peroxide (catalase activity), sucrose tolerance and the ability to grow at low oxygen levels (microaerobiosis), using the three following tests:

(1) For detection of catalase activity, a bacterial colony was taken from an axenic culture on TSA with a microbiology loop and was suspended in 3% hydrogen peroxide (Panreac). The appearance of bubbles was recorded as a positive result (Aslanzadeh, 2006).

(2) Sucrose tolerance was assessed by culturing isolates at room temperature for up to 7 days in transparent plastic vials containing LB broth supplemented with 0% (positive control), 10%, 20% and 30% sucrose (w/v, Sigma-Aldrich). The appearance of turbidity in the tubes with respect to negative controls (i.e. tubes containing no inoculated media) was recorded as a positive result. The range of sugar concentrations tested roughly corresponds with natural variation observed in floral nectars in wild South African plants (see above).

(3) Growth under microaerobiosis was determined by culturing isolates on TSA and incubating the plates at room temperature for 72 h in a candle jar. The appearance of colonies on the plates was recorded as a positive result.

### DNA isolation, PCR amplification and sequencing of the bacterial 16S rRNA gene

Genomic DNA was isolated by boiling bacterial colonies in 500 µL of ultrapure deionized water at 100 °C for 20 min. Cell debris was removed by centrifuging at 8000 g for 2 min.

The bacterial 16S rRNA gene was amplified using the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Reysenbach *et al.*, 2000) and the eubacterial-specific primer 27F (5'-AGAGTTTGTATCMTGGCTCAG-3', where M = A or C) (Braker *et al.*, 2001). Reaction mixtures contained 5 µL of NH<sub>4</sub> buffer (10×; Bionline, London, UK), 1 mM MgCl<sub>2</sub>, 0.4 µM of each primer (Sigma-Aldrich), 250 µM of each dNTP (Sigma-Aldrich), 3 U Biotaq DNA polymerase (Bionline) and 2–5 µL DNA extract. The final volume was adjusted to 50 µL with ultrapure deionized water. Amplification was carried out in a Flex-Cycler PCR thermal cycler (Analytik Jena, Jena, Germany) and consisted of a denaturation step of 4 min at 94 °C, followed by 35 cycles of 90 s at 94 °C, 90 s at 50 °C and 2 min at 72 °C, and a final extension at 72 °C for 2 min. PCR products were cleaned up with ExoSAP-IT (USB Corporation, Cleveland, OH), which degrades excess primers and nucleotides.

Sequencing of amplicons was performed 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 (5'-GTGCCAGCMGCCGCGGTAA-3', where M = A or C), 906F (5'-GAAACTTAAAKGAATTG-3'), 519R (5'-GWATTACCGCGGCKGCTG-3', where W = A or T and

Table 2. Bacterial OTUs recovered from nectar samples of South African plants\*†

Plant species†	OTUs (no.)	<i>Pseudomonas</i> sp.	<i>Pantoea</i> sp.	<i>Burkholderia</i> sp.	<i>Asaia</i> sp.	<i>Leftsonia</i> sp.	Micrococaceae	<i>Bacillus</i> sp. A	Enterobacteriaceae	<i>Methylobacterium</i> sp. A	Microbacteriaceae	<i>Sphingomonadaceae</i> sp. A	Alcaligenaceae	<i>Bacillus</i> sp. B	<i>Methylobacterium</i> sp. B	<i>Paenibacillus</i> sp.	<i>Sphingomonadaceae</i> sp. B	<i>Stenotrophomonas</i> sp.	Xanthomonadaceae	
<i>Adhatoda andromeda</i>	2	2																		
<i>Ajuga ophyndis</i>	2	1				1														
<i>Burchella bubalina</i>	2		1																	1
<i>Cycnium adonense</i>	10	2		1				1	1		2			1		1				
<i>Cycnium racemosum</i>	5	1		1		1						1	1							
<i>Disa crassicornis</i>	3	2	1																	
<i>Erica cerinthoides</i>	1																			
<i>Eriosema distinctum</i>	1	1																		
<i>Freesia laxa</i>	3		1	1													1			
<i>Gladiolus longicollis</i>	1									1										
<i>Gladiolus parvulus</i>	3		1	1		1														
<i>Glumicalyx godeloides</i>	2									1					1					
<i>Graderia scabra</i>	1																			
<i>Haemanthus humilis</i>	1		1																	
<i>Kniphofia caulescens</i>	3		1																	
<i>Kniphofia sp.</i>	2		1																	
<i>Moraea unibracteata</i>	2	2																		
<i>Protea caffra</i>	4			1	2				1											

Table 2. Continued

Plant species <sup>†</sup>	OTUs (no.)	<i>Pseudomonas</i> sp.	<i>Pantoea</i> sp.	<i>Burkholderia</i> sp.	<i>Asaia</i> sp.	<i>Leifsonia</i> sp.	<i>Micrococccaceae</i>	<i>Bacillus</i> sp. A	<i>Enterobacteriaceae</i>	<i>Methylobacterium</i> sp. A	<i>Microbacteriaceae</i>	<i>Sphingomonadaceae</i> sp. A	<i>Alcigenaceae</i>	<i>Bacillus</i> sp. B	<i>Methylobacterium</i> sp. B	<i>Paenibacillus</i> sp.	<i>Sphingomonadaceae</i> sp. B	<i>Stenotrophomonas</i> sp.	<i>Xanthomonadaceae</i>	
<i>Ruellia cordata</i>	2	1				1														
<i>Silene bellidioides</i>	2		2																	
<i>Thunbergia natalensis</i>	1			1																
Total	53	12	9	6	3	3	3	2	2	2	2	2	1	1	1	1	1	1	1	1

<sup>†</sup>To avoid confusion, different OTUs belonging to the same bacterial taxon were named as sp. A and sp. B.

<sup>‡</sup>Except in the first column, figures refer to the number of nectar samples from a given plant species from which the corresponding OTU<sub>0.03</sub> was recovered. The first column shows the total number of bacterial isolates recovered per plant species.

<sup>§</sup>Only those plant species from which nectar bacteria were isolated are included.

K = G or T), 907R (5'-CCGTCAATTCCTTTTRAGTTT-3', where R = A or G) and 1492R (Reysenbach *et al.*, 2000). The sequences were determined on an automated sequencer (ABI Prism 3130xl; Applied Biosystems), and assembled and manually edited with the program SEQUENCHER ver. 4.9 (Gene Codes Corp., Ann Arbor, MI). The GenBank accession numbers of the DNA sequences obtained in this study are JN872496–JN872548 (for further details see Supporting information, Table. S1).

## Data analyses

The 16S rRNA gene sequences obtained from nectar bacteria were compared with reference sequences from the GenBank databases, using BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the Ribosomal Database Project (RDP) website (<http://rdp.cme.msu.edu/>). Isolates were assigned to genus or the highest taxonomic rank possible, leaving further hierarchical taxonomy unidentified.

16S rRNA gene sequences were included in a multiple alignment generated by CLUSTALW (Chenna *et al.*, 2003) and the resulting alignment was trimmed by Gblocks (Castresana, 2000) to eliminate poorly aligned positions and divergent regions. A phylogenetic tree was constructed for nectar isolates and reference sequences using MRBAYES v3.1.2 (Ronquist & Huelsenbeck, 2003) as implemented on the CIPRES Science Gateway (Miller *et al.*, 2010). The simplest model of sequence evolution among those available in MRBAYES that best fits the sequence data was determined using the Akaike Information Criterion. This test was conducted using the JMODELTEST 0.1.1 package (Posada, 2008), and resulted in selection of a general time-reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites (GTR + G + I). Four chains were run twice (chain temperature = 0.2; sample frequency = 100) until average standard deviation of split frequencies was below 0.01 (*c.* 9.2 million generations). A 50% majority-rule consensus tree was calculated using the *sumt* command and discarding the first 25% of the trees to yield the final Bayesian estimate of phylogeny. The resulting tree was finally drawn and further edited with TREEGRAPH2 (Stöver & Müller, 2010).

Determination of the number of distinct operational taxonomic units (OTUs) occurring in our set of DNA sequences and assignment of sequences to OTUs was done with the program MOTHUR v.1.17.3 (Schloss *et al.*, 2009). DNA dissimilarity cut-offs of 1% and 3% were used in these analyses. To assess the overall richness of bacterial OTUs, sample-based rarefaction methods were applied to presence-absence data. Due to the limited number of nectar samples available in this work, OTU

occurrence data from all samples were analysed together (i.e. irrespective of the plant species and/or family of origin). Average rarefaction curves were computed with the ESTIMATES v.8.2.0 program (Colwell, 2009), using 50 randomizations and sampling without replacement. As our data are based on incidence, the ICE and Chao2 nonparametric estimators of the expected OTU richness were also calculated.

## Results

The results of the microbiological analysis of nectar samples are provided in Tables 1 and 2. A total of 53 bacterial isolates were recovered from 38 nectar samples (53.5%,  $n = 71$ ), and 21 of the plant species surveyed (77.8%,  $n = 27$ ). No bacteria were recovered from *D. luteo-albidum*, *G. appendiculatus*, *P. roupelliae*, *S. aethiopica*, *W. lepidia* or *W. pillansii*. All bacterial isolates recovered from nectar were able to grow under microaerobiosis and showed a positive reaction in the catalase test (Table 3). Most isolates also tolerated 10–30% (w/v) sucrose, the exceptions being some isolates from the genera *Burkholderia* and *Methylobacterium*, and

the families *Sphingomonadaceae* and *Xanthomonadaceae* (Table 3).

In the MOTHUR analysis of the DNA sequence data for bacterial isolates, 18 OTUs were identified at the 3% dissimilarity cut-off (OTU<sub>0.03</sub>; Table 2). Only six additional OTUs were identified when the dissimilarity cut-off was lowered to 1%, thus giving a total of 24 OTU<sub>0.01</sub>. Given the small difference in total OTUs obtained with the two thresholds, only OTU<sub>0.03</sub> were retained for subsequent analyses, as this represents the threshold commonly used to distinguish bacterial OTUs at the species level in environmental studies (Lambais *et al.*, 2006; Teixeira *et al.*, 2010).

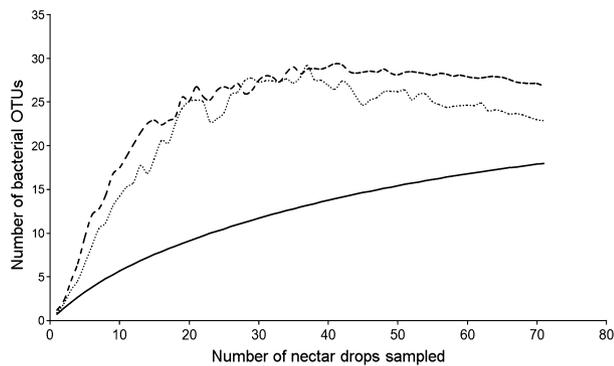
When rarefaction-based methods were applied to obtain reliable estimates of total bacterial OTU richness, the OTU<sub>0.03</sub> rarefaction curve was close to reaching a plateau for the number of nectar samples examined ( $n = 71$ ; Fig. 1). Although additional OTUs are expected to appear by further increasing the sampling effort (and/or lowering the DNA dissimilarity cut-off; see Fig. S1), results of this survey seem to provide a reliable basis for estimating overall bacterial OTU richness in the floral nectar of the set of plants surveyed. OTU<sub>0.03</sub> richness estimates were 26.8

**Table 3.** Physiological characteristics of nectar bacterial isolates

Taxonomical affiliation of OTU <sub>0.03</sub> *	Growth under microaerobiosis	Catalase production	Sucrose tolerance		
			10%	20%	30%
<i>Actinobacteria</i>					
<i>Leifsonia</i> sp. (3)	+	+	+	+	+
<i>Microbacteriaceae</i> (2)	+	+	+	+	+
<i>Micrococcaceae</i> (3)	+	+	+	+	+
<i>Firmicutes</i>					
<i>Bacillus</i> sp. A (2)	+	+	+	+	+
<i>Bacillus</i> sp. B (1)	+	+	+	+	+
<i>Paenibacillus</i> sp. (1)	+	+	+	+	+
<i>Proteobacteria</i>					
<i>Alphaproteobacteria</i>					
<i>Asaia</i> sp. (3)	+	+	+	+	+
<i>Methylobacterium</i> sp. A (2)	+	+	+	+	V
<i>Methylobacterium</i> sp. B (1)	+	+	+	–	–
<i>Sphingomonadaceae</i> sp. A (2)	+	+	V	V	–
<i>Sphingomonadaceae</i> sp. B (1)	+	+	+	–	–
<i>Betaproteobacteria</i>					
<i>Alcaligenaceae</i> (1)	+	+	+	+	+
<i>Burkholderia</i> sp. (6)	+	+	+	+	V
<i>Gammaproteobacteria</i>					
<i>Enterobacteriaceae</i> (2)	+	+	+	+	+
<i>Pantoea</i> sp. (9)	+	+	+	+	+
<i>Pseudomonas</i> sp. (12)	+	+	+	+	+
<i>Stenotrophomonas</i> sp. (1)	+	+	+	+	+
<i>Xanthomonadaceae</i> (1)	+	+	+	+	–

+, positive; –, negative; V, variable.

\*The number of isolates belonging to each OTU<sub>0.03</sub> is given in parentheses. As in Table 2, to avoid confusion, different OTUs belonging to the same family or genus were named as sp. A and sp. B.



**Fig. 1.** Graphical representation of the rarefaction curve (solid line) and nonparametric estimators of nectar bacteria OTU<sub>0.03</sub> richness for our dataset: ICE (long dashes) and Chao2 (dotted line).

(ICE estimator) and 22.9 species (Chao2 estimator; Fig. 1). Therefore, our sampling recovered more than 67% of the estimated number of bacterial OTUs occurring in the nectar of sampled plant species in the study area.

Phylogenetic analyses showed a distribution of isolates among three major bacterial phyla: *Actinobacteria*, *Firmicutes* and *Proteobacteria* (*Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*) (Table 2 and Fig. 2), the last-named being the most frequent (77.4% of isolates). Furthermore, *Pseudomonas* and *Pantoea* were the two most common bacterial genera recovered, albeit with a low overall incidence (16.9% and 12.7% of nectar samples analysed, respectively). Other *Proteobacteria* genera identified in phylogenetic analysis were *Asaia*, *Burkholderia*, *Methylobacterium*, *Stenotrophomonas*, and several other representatives from the families *Alcaligenaceae*, *Enterobacteriaceae*, *Sphingomonadaceae* and *Xanthomonadaceae*. On the other hand, 15.1% of bacterial isolates belonged to the phylum *Actinobacteria*, and were classified within the families *Microbacteriaceae* (including *Leifsonia* sp.) and *Micrococcaceae*. Finally, members of the phylum *Firmicutes* comprised only a negligible fraction of isolates (7.5%), and belonged to the genera *Bacillus* and *Paenibacillus*.

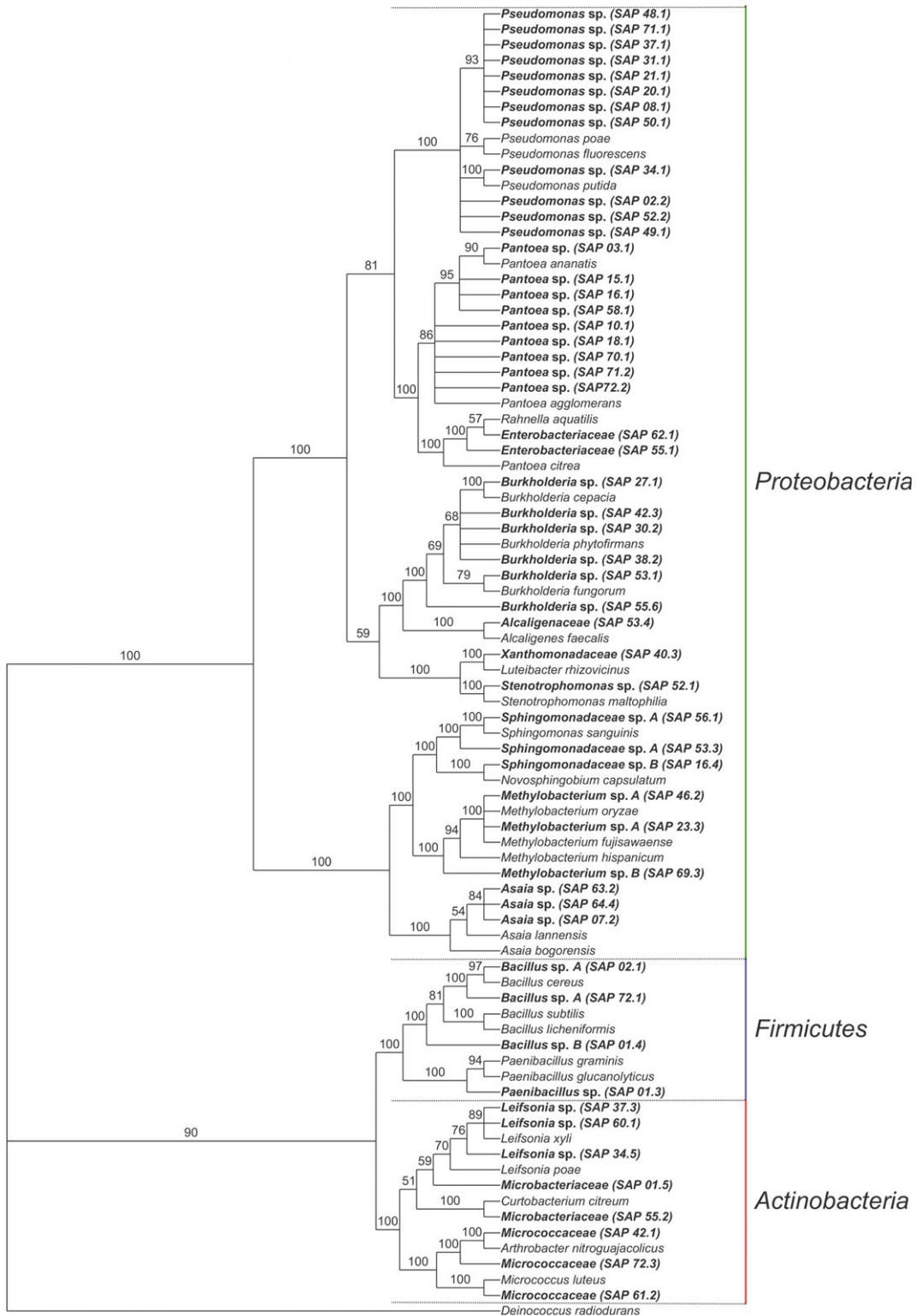
## Discussion

We have presented here the first analysis of bacterial communities associated with floral nectar in a diverse array of wild plants, which represents a necessary step towards a better understanding of multikingdom interactions surrounding insect-pollinated flowers in nature. We focus on nectar, the main reward offered by plants to their pollinators, which is considered here the key floral resource in supporting a bacterial microcosm. This is in sharp contrast to previous investigations which either focus on the bacterial communities inhabiting other floral

parts (Junker *et al.*, 2011) or do not provide details on the floral organs from which the microbiota was sampled (Yamada *et al.*, 2000; Lachance *et al.*, 2003; Yukphan *et al.*, 2004).

A main finding from the present study was that bacteria are relatively common inhabitants of floral nectar of animal-pollinated South African plants, being present in 21 plant species and more than a half of samples analysed. This picture is similar to that encountered by Ehlers & Olesen (1997) in *Epipactis helleborine* at different locations in northern Europe. Unfortunately, there is no additional information on the prevalence of bacteria in nectar of wild plants.

On the other hand, the studied bacterial microbiota associated with floral nectar was characterized by relatively low species richness. Eighteen bacterial OTUs were identified in MOTHUR-based analyses at the 3% dissimilarity cut-off. Lowering this cut-off to 1% allowed the identification of six additional OTUs, which did not have a dramatic impact on rarefaction-based estimates of total bacterial OTU richness (see supporting Appendix S1 and Fig. S1). Furthermore, although it is expected that additional sampling effort would increase the number of OTUs identified, rarefaction analysis revealed that, for the group of plants sampled, we recovered a high proportion of the OTU richness of nectar-inhabiting bacteria. A similar low value for species richness has been reported for nectar yeast communities (Pozo *et al.*, 2011), but contrasts with the high species richness of other plant-associated environments, such as the rhizosphere (Teixeira *et al.*, 2010; Weinert *et al.*, 2011) or the phyllosphere (Lambais *et al.*, 2006). Along the same line, nectar bacterial communities were characterized by a moderate phylogenetic diversity, as the isolates belonged to only three different bacterial phyla: *Proteobacteria*, *Actinobacteria* and *Firmicutes*. Within the *Proteobacteria*, nectar bacteria were distributed among the *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. A considerable proportion of isolates were members of the *Gammaproteobacteria*, with *Pseudomonas* and *Pantoea* being the predominant genera. Low phylogenetic diversity is also a characteristic of nectar-associated yeast communities (Brysch-Herzberg, 2004; Herrera *et al.*, 2010; Pozo *et al.*, 2011; de Vega & Herrera, 2012), a concordance which stresses the potential role of nectar as a habitat filter that excludes species that do not possess habitat-specific physiological adaptations. Interestingly, although we found no dominant bacterial species in the set of nectar samples studied, one single yeast species (*Metschnikowia reukaufii*) has been repeatedly isolated from nectar at different locations (Eisikowitch *et al.*, 1990; Brysch-Herzberg, 2004; Herrera *et al.*, 2010; Pozo *et al.*, 2011; de Vega & Herrera, 2012).



**Fig. 2.** Phylogenetic relationship of 16S rRNA gene sequences from nectar bacteria retrieved in this study from South African plants (indicated in bold type and with collection reference numbers) and reference sequences of type strains stored in the GenBank database, as determined by Bayesian inference. *Deinococcus radiodurans* served as the outgroup for this analysis. GenBank accession numbers and further details on nectar isolates and reference strains are provided in Table S1. Numbers above branches show clade credibility values (posterior probabilities).

Apart from the predominance of *Gammaproteobacteria* in the nectar from South African plants reported in this work, members of this class have been also identified as the dominant bacterial inhabitants of other plant-associated environments, such as the surface of leaves (Ercolani, 1991; Thompson *et al.*, 1993; Krimm *et al.*, 2005; Junker *et al.*, 2011) and petals (Junker *et al.*, 2011), the interior of pitchers of some carnivorous plants (Siragusa *et al.*, 2007; Koopman *et al.*, 2010), and sugar-rich sap exudates (Lagacé *et al.*, 2004, 2006; Filteau *et al.*, 2010). However, while *Pseudomonas* was the most prevalent genus among nectar isolates (Table 2) and has been repeatedly identified as a key component of epiphytic bacterial communities on leaves (Ercolani, 1991; Thompson *et al.*, 1993; Krimm *et al.*, 2005; Junker *et al.*, 2011), the results from a recent investigation show that the surface of petals of some plant species are predominantly colonized by members of the family *Enterobacteriaceae* (Junker *et al.*, 2011). Nevertheless, in the present work we have only identified bacterial isolates up to genus level, which precludes further species-based comparisons of microbial community composition with previous reports. Furthermore, as nectar-inhabiting and flower epiphytic microbial communities have not been yet extensively characterized, and intrafloral microhabitat heterogeneity remains poorly understood, a detailed comparative analysis of floral nectar and petals as microbial habitats cannot be performed.

Nearly all bacteria isolated from floral nectar in this study appear to be physiologically able to overcome the three main stressors characteristic of their habitat, namely high osmotic pressure, low oxygen levels and presence of toxic hydrogen peroxide. Although the physiological mechanisms allowing survival of nectar microorganisms in this sugar-rich habitat have not been studied in detail to date, they could be similar to those employed for coping with the osmotic stress imposed by high levels of solutes in other environments such as salterns, hypersaline lakes, and salty or sugary food products (reviewed by Beales, 2004; Grant, 2004). The ability of nectar bacteria to grow at low oxygen levels might be relevant in situations where oxygen diffusion through nectaries is hindered (e.g. in plants with relatively long horn-shaped nectaries, where a biofilm usually appears at the air-nectar interface; C.M. Herrera, pers. obs.) or when environmental oxygen is depleted by microbial metabolism. Additionally, catalase activity might protect nectar bacteria from the toxic action of H<sub>2</sub>O<sub>2</sub>, as demonstrated for other plant-associated microbes (Xu & Pan, 2000). Nevertheless, it has also been noted that in some bacterial species the presence or absence of catalase is not correlated with the ability of the microorganism to overcome the lethal effects of H<sub>2</sub>O<sub>2</sub>, as susceptibility to this toxic compound

also depends on other factors (see, for example, Schwartz *et al.*, 1983; Wilson & Weaver, 1985). Moreover, Carter *et al.* (2007) demonstrated that some *Proteobacteria*, including strains of *Pseudomonas syringae* and *Pseudomonas fluorescens*, were sensitive to the H<sub>2</sub>O<sub>2</sub> concentrations observed in the floral nectar of ornamental *Nicotiana* species. Thus, the hypothesized relationship between catalase activity and survival in nectar clearly deserves further investigation.

Apart from the three stressors mentioned above, additional factors not considered in this study could limit microbial growth and/or survival in floral nectar. For example, it has been recently demonstrated that the antimicrobial activity of *Petunia hybrida* nectar is not based on H<sub>2</sub>O<sub>2</sub> production but on RNase activities (Hillwig *et al.*, 2010). Antimicrobial properties have also been attributed to a GDSL lipase of the floral nectar of *Jacaranda mimosifolia* (Kram *et al.*, 2008). This growing list of nectar proteins and secondary metabolites potentially implicated in plant antimicrobial defence (see also Adler, 2000) contrasts with the lack of information on the physiological strategies of nectar microbes for adapting to their stressful habitat (but see Herrera *et al.*, 2012, for a recent study on nectar yeasts). In any case, strong selective pressures are expected to turn nectar into a potential microorganism-free environment, operating over micro- and macroevolutionary time scales.

In summary, our results have shown that bacteria are common inhabitants of floral nectar of South African plants, and their communities are characterized by low species richness and moderate phylogenetic diversity. Moreover, we have identified osmotolerance, catalase activity and the ability to grow under microaerobiosis as traits that might help bacteria to overcome important factors limiting their survival and/or growth in nectar. Future work should clarify the role of bacteria within the plant–yeast–pollinator system and might help to fill a conspicuous gap in our knowledge of ecological interactions involving macro- and microorganisms at the intersection of several kingdoms.

## Acknowledgements

We thank Prof. S. D. Johnson for logistical support and hospitality and R. G. Albaladejo, H. Burger, V. Ferrero, J. Rodger, P. Rymer, A. Shuttleworth and S. Steenhuisen for field assistance. This work was supported by funds from the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (Proyecto de Excelencia P09-RNM-4517 to C.M. H.), and Ministerio de Ciencia e Innovación (Juan de la Cierva Programme to C.dV.). Beatriz Guzmán is gratefully acknowledged for her assistance with phylogenetic analyses.

## References

- Adler LS (2000) The ecological significance of toxic nectar. *Oikos* **91**: 409–420.
- Andrews JH & Harris RF (2000) The ecology and biogeography of microorganisms on plant surfaces. *Annu Rev Phytopathol* **38**: 145–180.
- Aslanzadeh J (2006) Biochemical profile-based microbial identification systems. *Advanced Techniques in Diagnostic Microbiology* (Tang Y-W & Stratton CW, eds), pp. 84–116. Springer, New York.
- Barton L (2005) *Structural and Functional Relationships in Prokaryotes*, pp. 529–601. Springer, New York.
- Beales N (2004) Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. *Compr Rev Food Sci Food Saf* **3**: 1–20.
- Bode HB (2009) Entomopathogenic bacteria as a source of secondary metabolites. *Curr Opin Chem Biol* **13**: 224–230.
- Braker G, Ayala-del-Río HL, Devol AH, Fesefeldt A & Tiedje JM (2001) Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. *Appl Environ Microbiol* **67**: 1893–1901.
- Brysch-Herzberg M (2004) Ecology of yeasts in plant–bumblebee mutualism in Central Europe. *FEMS Microbiol Ecol* **50**: 87–100.
- Canto A, Pérez R, Medrano M, Castellanos MC & Herrera CM (2007) Intra-plant variation in nectar sugar composition in two *Aquilegia* species (Ranunculaceae): contrasting patterns under field and glasshouse conditions. *Ann Bot* **99**: 653–660.
- Canto A, Herrera CM, Medrano M, Pérez R & García IM (2008) Pollinator foraging modifies nectar sugar composition in *Helleborus foetidus* (Ranunculaceae): an experimental test. *Am J Bot* **95**: 315–320.
- Carter C & Thornburg RW (2004) Is the nectar redox cycle a floral defense against microbial attack? *Trends Plant Sci* **9**: 320–324.
- Carter C, Healy R, O'Tool NM, Naqvi SM, Ren G, Park S, Beattie GA, Horner HT & Thornburg RW (2007) Tobacco nectaries express a novel NADPH oxidase implicated in the defense of floral reproductive tissues against microorganisms. *Plant Physiol* **143**: 389–399.
- Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* **17**: 540–552.
- 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. Available at: <http://purl.oclc.org/estimates>.
- de Vega C & Herrera CM (2012) Relationships among nectar-dwelling yeasts, flowers and ants: patterns and incidence on nectar traits. *Oikos*, in press.
- 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.
- Ehlers BK & Olesen JM (1997) The fruit-wasp route to toxic nectar in *Epipactis* orchids? *Flora* **192**: 223–229.
- Eisikowitch D, Kevan PG & Lachance MA (1990) The nectar-inhabiting yeasts and their effect on pollen germination in common milkweed, *Asclepias syriaca* L. *Isr J Bot* **39**: 217–225.
- Ercolani GL (1991) Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. *Microb Ecol* **21**: 35–48.
- Filteau M, Lagacé L, LaPointe G & Roy D (2010) Seasonal and regional diversity of maple sap microbiota revealed using community PCR fingerprinting and 16S rRNA gene clone libraries. *Syst Appl Microbiol* **33**: 165–173.
- Gilliam M, Moffett JO & Kauffeld NK (1983) Examination of floral nectar of *Citrus*, cotton, and Arizona desert plants for microbes. *Apidologie* **14**: 299–302.
- Grant WD (2004) Life at low water activity. *Proc R Soc Lond B Biol Sci* **359**: 1249–1267.
- Heil M (2011) Nectar: generation, regulation and ecological functions. *Trends Plant Sci* **16**: 191–200.
- Herrera CM & Pozo MI (2010) Nectar yeasts warm the flowers of a winter-blooming plant. *Proc R Soc Lond B Biol Sci* **277**: 1827–1834.
- 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 Lond B Biol Sci* **277**: 747–754.
- Herrera CM, Pozo MI & Bazaga P (2012) Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. *Mol Ecol*, doi:10.1111/j.1365-294X.2011.05402.x.
- Hillwig MS, Liu X, Liu G, Thornburg RW & Macintosh GC (2010) Petunia nectar proteins have ribonuclease activity. *J Exp Bot* **61**: 2951–2965.
- Junker RR, Loewel C, Gross R, Dötterl S, Keller A & Blüthgen N (2011) Composition of epiphytic bacterial communities differs on petals and leaves. *Plant Biol* **13**: 918–924.
- Kent AD & Triplett EW (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annu Rev Microbiol* **56**: 211–236.
- Koopman MM, Fuselier DM, Hird S & Carstens BC (2010) The carnivorous pale pitcher plant harbours diverse, distinct, and time-dependent bacterial communities. *Appl Environ Microbiol* **76**: 1851–1860.

- Kram BW, Bainbridge EA, Perera MA & Carter C (2008) Identification, cloning and characterization of a GDSL lipase secreted into the nectar of *Jacaranda mimosifolia*. *Plant Mol Biol* **68**: 173–183.
- Krimm U, Abanda-Nkpawatt D, Schwab W & Schreiber L (2005) Epiphytic microorganisms on strawberry plants (*Fragaria ananassa* cv. Elsanta): identification of bacterial isolates and analysis of their interaction with leaf surfaces. *FEMS Microbiol Ecol* **53**: 483–492.
- Lachance MA, Bowles JM & Starmer WT (2003) *Metschnikowia santaceciliae*, *Candida hawaiiiana*, and *Candida kipukae*, three new yeast species associated with insects of tropical morning glory. *FEMS Yeast Res* **3**: 97–103.
- Lagacé L, Pitre M, Jacques M & Roy D (2004) Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing. *Appl Environ Microbiol* **70**: 2052–2060.
- Lagacé L, Jacques M, Mafu AA & Roy D (2006) Compositions of maple sap microflora and collection system biofilms evaluated by scanning electron microscopy and denaturing gradient gel electrophoresis. *Int J Food Microbiol* **109**: 9–18.
- Lambais MR, Crowley DE, Cury JC, Büll RC & Rodrigues RR (2006) Bacterial diversity in tree canopies of the Atlantic forest. *Science* **312**: 1917.
- Latour X & Lemañeau P (1997) Métabolisme carboné et énergétique des *Pseudomonas* spp. fluorescents saprophytes à oxydase positive. *Agronomie* **17**: 427–443.
- Long SR (1996) *Rhizobium* symbiosis: nod factors in perspective. *Plant Cell* **8**: 1885–1898.
- Miller MA, Pfeiffer W & Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop (GCE)*, pp. 1–8. New Orleans, LA.
- Nicolson SW & Thornburg RW (2007) Nectar chemistry. *Nectaries and Nectar* (Nicolson SW, Nepi M & Pacini E, eds), pp. 215–264. Springer-Verlag, Dordrecht.
- Park S & Thornburg RW (2009) Biochemistry of nectar proteins. *J Plant Biol* **52**: 27–34.
- Peay KG, Belisle M & Fukami T (2012) Phylogenetic relatedness predicts priority effects in nectar yeast communities. *Proc R Soc Lond B Biol Sci* **279**: 749–758.
- Posada D (2008) jModelTest: phylogenetic model averaging. *Mol Biol Evol* **25**: 1253–1256.
- Pozo MI, Herrera CM & Bazaga P (2011) Species richness of yeast communities in floral nectar of southern Spanish plants. *Microb Ecol* **61**: 82–91.
- 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.
- Ronquist F & Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.
- 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.
- Schwartz CE, Krall J, Norton L, McKay K, Kay D & Lynch RE (1983) Catalase and superoxide dismutase in *Escherichia coli*. *J Biol Chem* **258**: 6277–6281.
- Siragusa AJ, Swenson JE & Casamatta DA (2007) Culturable bacteria present in the fluid of the hooded-pitcher plant *Sarracenia minor* based on 16S rRNA gene sequence data. *Microb Ecol* **54**: 324–331.
- Smith SE & Read DJ (2008) *Mycorrhizal Symbiosis*, 3rd edn. Academic Press, London.
- Stevens PF (2011) Angiosperm phylogeny website, version 9. Available at: <http://www.mobot.org/MOBOT/research/APweb/> (last accessed 21 October 2011).
- Stöver BC & Müller KF (2010) TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics* **11**: 7.
- Ström K, Sjögren J, Broberg A & Schnürer J (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl Environ Microbiol* **68**: 4322–4327.
- 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.
- Thompson IP, Bailey MJ, Fenlon JS *et al.* (1993) Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugar beet (*Beta vulgaris*). *Plant Soil* **150**: 177–191.
- Weinert N, Piceno Y, Ding GC, Meincke R, Heuer H, Berg G, Schloter M, Andersen G & Smalla K (2011) PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: many common and few cultivar-dependent taxa. *FEMS Microbiol Ecol* **75**: 497–506.
- Wilson CB & Weaver WM (1985) Comparative susceptibility of group B streptococci and *Staphylococcus aureus* to killing by oxygen metabolites. *J Infect Dis* **152**: 323–329.
- Xu XQ & Pan SQ (2000) An *Agrobacterium* catalase is a virulence factor involved in tumorigenesis. *Mol Microbiol* **35**: 407–414.
- Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, Saono S, Seki T, Uchimura T & Komagata K (2000) *Asaia bogorensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the  $\alpha$ -Proteobacteria. *Int J Syst Evol Microbiol* **50**: 823–829.
- Yukphan P, Potacharoen W, Tanasupawat S, Tanticharoen M & Yamada Y (2004) *Asaia krungthepensis* sp. nov., an acetic acid bacterium in the  $\alpha$ -Proteobacteria. *Int J Syst Evol Microbiol* **54**: 313–316.

**Note added in proof:**

During the pre-print processing of the present work, the authors became aware of the publication of an article addressing the study of nectar bacterial communities in three species of cultivated plants (Fridman *et al.*, 2012. Bacterial communities in floral nectar. *Environmental Microbiology Reports*, 4(1):97-104.)

**Supporting Information**

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

**Fig. S1.** Graphical representation of the rarefaction curve (solid line) and nonparametric estimators of nectar bacte-

ria OTU<sub>0.01</sub> richness for our dataset: ICE (long dash) and Chao2 (dotted line).

**Table S1.** GenBank accession numbers of 16S rRNA gene sequences and further details of nectar isolates and reference strains included in our phylogenetic analysis.

**Appendix S1.** Rarefaction based analysis of OTU<sub>0.01</sub> richness.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.