PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2010) **277**, 747–754
doi:10.1098/rspb.2009.1485
Published online 4 November 2009

Inhospitable sweetness: nectar filtering of pollinator-borne inocula leads to impoverished, phylogenetically clustered yeast communities

Carlos M. Herrera[1,*], Azucena Canto[2], María I. Pozo[1] and Pilar Bazaga[1]

[1]*Estación Biológica de Doñana, CSIC, Américo Vespucio s/n, E-41092 Sevilla, Spain*
[2]*Centro de Investigación Científica de Yucatán (CICY), A.C. Calle 43 No. 130 Chuburná de Hidalgo 97200, Mérida, Yucatán, México*

Identifying the rules and mechanisms that determine the composition and diversity of naturally co-occurring species assemblages is a central topic in community ecology. Although micro-organisms represent the 'unseen majority' of species, individuals and biomass in many ecosystems and play pivotal roles in community development and function, the study of the factors influencing the assembly of microbial communities has lagged behind that of plant and animal communities. In this paper, we investigate experimentally the mechanisms accounting for the low species richness of yeast communities inhabiting the nectar of the bumble-bee-pollinated *Helleborus foetidus* (Ranunculaceae), and explore the relationships between community assembly rules and phylogenetic relatedness. By comparing yeast communities on the glossae of foraging bumble-bees (the potential species pool) with those eventually establishing in virgin nectar probed with bee glossae (the realized community), we address the questions: (i) does nectar filter yeast inocula, so that the communities eventually established there are not random subsamples of species on bumble-bee glossae? and (ii) do yeast communities establishing in *H. foetidus* nectar exhibit some phylogenetic bias relative to the species pool on bumble-bee glossae? Results show that nectar filtering leads to species-poor, phylogenetically clustered yeast communities that are a predictable subset of pollinator-borne inocula. Such strong habitat filtering is probably due to *H. foetidus* nectar representing a harsh environment for most yeasts, where only a few phylogenetically related nectar specialists physiologically endowed to tolerate a combination of high osmotic pressure and fungicidal compounds are able to develop.

Keywords: community assembly rules; environmental filtering; *Helleborus foetidus*; *Metschnikowia*; phylogenetic structure; nectar yeast communities

1. INTRODUCTION

Identifying the rules and mechanisms that determine the composition and diversity of co-occurring species assemblages is a focal topic in community ecology. Current knowledge on these aspects, however, is largely confined to animal and plant communities (Begon *et al.* 2006; Gurevitch *et al.* 2006; Emerson & Gillespie 2008; Vamosi *et al.* 2009), possibly because practical difficulties (e.g. species identification) have until recently hindered the progress of microbial community ecology (Fierer *et al.* 2007; Jackson *et al.* 2007). Micro-organisms, however, represent the 'unseen majority' of species, individuals and biomass in many ecosystems, and play pivotal roles in community development and function (Whitman *et al.* 1998). The structure of microbial communities has important functional implications in ecosystems (Fuhrman 2009), thus elucidating the rules and mechanisms involved in the assembly of microbial communities will be essential to a better understanding of aspects of ecosystem functioning in which micro-organisms are involved (Horner-Devine & Bohannan 2006; Horner-Devine *et al.* 2007; Maherali & Klironomos 2007; Bryant *et al.* 2008; Fuhrman 2009).

Ecological studies on microbial communities have mostly focused on hyperdiverse microbiota associated with soil, plant surfaces and aquatic environments (Lambais *et al.* 2006; Schloss & Handelsman 2006*b*; Fierer *et al.* 2007; Peay *et al.* 2008; Shaw *et al.* 2008). The formidable complexity of these systems, however, limits the possibilities of unravelling the mechanisms influencing microbial composition and diversity at biologically relevant spatial scales in the field. Such questions could be profitably addressed in 'scaled-down' natural microbial communities made up of relatively few, easily identifiable species confined to well-defined microhabitats amenable to experimental manipulation (Maherali & Klironomos 2007). Dense yeast communities often occur in the floral nectar of animal-pollinated plants, where they can behave as parasites of plant-pollinator mutualisms (Brysch-Herzberg 2004; Canto *et al.* 2008; Herrera *et al.* 2008, 2009). Yeast communities of floral

* Author for correspondence (herrera@cica.es).

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2009.1485 or via http://rspb.royalsocietypublishing.org.

Received 15 August 2009
Accepted 6 October 2009

nectar seem to be characterized by lower species richness (Vörös-Felkai 1957; Eisikowitch *et al.* 1990; Brysch-Herzberg 2004) than those occurring in other environments like soil, plant surfaces or invertebrate guts (Lachance *et al.* 2001; Suh *et al.* 2005; Connell *et al.* 2008). In addition, species concepts and systematics are reasonably well developed for yeasts (Barnett *et al.* 2000), further contributing to render nectar yeast communities a manageable study system, particularly suited to address detailed ecological questions that are hard to tackle in more complex microbiota.

In this paper, we adopt an experimental approach to investigate the mechanisms responsible for the low species richness of yeast communities inhabiting the floral nectar of *Helleborus foetidus* (Ranunculaceae), a bumble-bee-pollinated plant, and to explore the possible relationships between community assembly mechanisms and phylogenetic patterns. Yeasts colonize *H. foetidus* floral nectar following probing of the nectaries by foraging bumble-bees that carry inocula on their glossae (Brysch-Herzberg 2004; Canto *et al.* 2008). Consequently, the potential composition of yeast communities in the nectar can be determined by examining the composition of yeast inocula that are 'travelling' on the glossae of foraging bumble-bees (i.e. the potential species pool). We will compare the yeast communities on bumble-bees' glossae with those becoming established in nectar (i.e. the realized community) previously inoculated by probing with bee glossae. This allows us to answer whether yeast communities in experimentally inoculated nectar mirror those on bumble-bee glossae or, alternatively, nectar filter the immigrant inocula so that a different community eventually builds up there. Environmental filtering has been suggested to result in phylogenetically clustered communities, i.e. made up of taxa that are more closely related among themselves than expected by chance (Webb *et al.* 2002; Horner-Devine & Bohannan 2006; Vamosi *et al.* 2009). This hypothesis motivates the second question addressed in this paper: do yeast communities experimentally established in *H. foetidus* nectar exhibit some phylogenetic bias in relation to the species pool on bumble-bee glossae?

In addition to shedding light on the mechanisms associated with the assembly of microbial communities, our study will also contribute to a better understanding of the interactions between plants and nectar yeasts, an intriguing relationship whose ecological and evolutionary significance is still far from being well established (Eisikowitch *et al.* 1990; Herrera *et al.* 2008, 2009). Recent quantitative surveys have documented very high frequencies of occurrence and extraordinary population densities of yeasts in nectar of animal-pollinated plants from three continents (Brysch-Herzberg 2004; Herrera *et al.* 2009; de Vega *et al.* 2009). Nectar yeasts, particularly at high densities, induce metabolic degradation of nectar, which can be detrimental to plant reproduction through reduced pollinator service (Herrera *et al.* 2008). This might originate selective pressures on plants to defend their nectars from exploiters through, e.g. antimicrobial secondary compounds (Adler 2000; Irwin *et al.* 2004). This so-called 'antimicrobial hypothesis' would explain the seemingly paradoxical presence of toxic substances in floral nectar as a defensive adaptation against nectarivorous microbes (González-Teuber & Heil 2009). The hypothesis relies on the as-yet-untested assumption that nectars with toxic substances are actually defended from nectarivorous microbes. By showing that nectar can be a poor growing place from many incoming yeasts, our study provides evidence supporting the antimicrobial hypothesis.

2. MATERIAL AND METHODS

(a) *Field and laboratory methods*

The diversity and composition of yeast communities naturally occurring in the floral nectar of *H. foetidus*, an early-blooming, bumble-bee-pollinated herb whose nectar harbours dense yeast populations (Herrera *et al.* 2008), was studied in 2008 at one locality of Sierra de Cazorla, south-eastern Spain ('Las Navillas', 1220 m elevation). On 25 February and 10 March, single-nectary nectar samples were collected with sterile microcapillaries from flowers of 10 widely spaced *H. foetidus* plants exposed to natural pollinator visitation (two flowers per plant, two nectaries per flower, on each collection date). A 1 µl aliquot of each nectar sample ($n = 80$) was streaked onto a Yeast Mould (YM) + chloramphenicol agar plate (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2.0% agar, 0.01% chloramphenicol, pH = 6.0), and incubated at 25°C. For each cultured nectar sample, distinct yeast isolates were obtained from all the resulting colonies following standard methods and criteria described in Yarrow (1998). For each isolate, approximately 500 nucleotides of the D1/D2 domain of the 26S subunit ribosomal DNA, the gene most commonly used for yeast identification and phylogenetic studies, were two-way sequenced following methods in Kurtzman & Robnett (1998) and Lachance *et al.* (1999). DNA sequences were obtained for a total of 39 yeast isolates, which were analysed following the methods described in §2*b*. The rest of each nectar sample (mean ± s.d. = 1.6 ± 0.6 µl, $n = 80$) was examined microscopically and yeast cell density determined following methods described by Herrera *et al.* (2009).

During the study period, the only species flowering locally were *H. foetidus* and *Rosmarinus officinalis* (Lamiaceae), and bumble-bees visited flowers of both species when growing at close range. To determine as comprehensively as possible the composition of the yeast species pool potentially arriving at *H. foetidus* flowers, 45 bumble-bees (*Bombus terrestris*) were hand-netted between 23 February and 17 March 2008 while foraging at flowers of *H. foetidus* ($n = 30$) and *R. officinalis* ($n = 15$) within a radius of approximately 1.5 km around the Las Navillas site. Netting was not done on the same *H. foetidus* plants used for sampling natural nectar yeast communities to avoid interference with other studies. Because we were interested in the overall composition of the local bumble-bee-borne yeast community, all netted bees were treated in the analyses as a single sample irrespective of the plant where they had been captured. Nevertheless, to test for the robustness of results to differences in the origin of samples, some analyses were repeated separately for data from bumble-bees collected from *H. foetidus* and *R. officinalis* plants.

Immediately after capture, bees were placed individually in sterile containers and anaesthetized by placing them inside a refrigerator at 4°C until used in the experiments, generally within a few hours of collection. In these experiments, bees were used as sources of inocula to generate

two types of artificial yeast communities, by inoculating either sterile yeast culture media ('bee-only communities' hereafter) or virgin natural nectar from *H. foetidus* flowers ('bee + nectar communities' hereafter) from 10 plants that had been previously excluded from pollinator visitation by bagging inflorescences at the bud stage. Since individual *H. foetidus* plants may differ in the susceptibility of nectar to yeast growth (C. M. Herrera 2009, unpublished data), two or three virgin flowers were collected from each plant, pooled in a container and a random sample of nectaries drawn for use in the inoculation experiments. The glossa of each individual bee was extended using fine forceps beyond the tip of the maxillary galeae and, depending on the treatment, either rubbed against the surface of a YM + chloramphenicol agar culture plate ('bee-only' treatment) or briefly introduced into a nectary of a virgin flower containing natural nectar to mimic natural nectar probing ('bee + nectar' treatment). The forceps used to handle bee tongues were cleaned between assays using 99 per cent ethanol. Bumblebee individuals were assigned to experimental treatments in one of two ways. Each bee was used either to inoculate culture media ($n = 15$) or nectar ($n = 15$) alone, or to inoculate both culture media and nectar in succession ($n = 15$), the order being alternated in successive assays. Results obtained with the two methods were similar and have been combined for the analyses. Culture plates from the bee-only treatment were handled as described above for wild-collected nectar samples. Inoculated nectaries (bee + nectar treatment) were kept within a sealed container at room temperature for 48 h, and then the nectar in each nectary was streaked onto a YM + chloramphenicol agar plate, incubated and yeast isolates obtained from each nectar sample following the same protocols as for cultures from wild-collected nectar and the bee-only treatment. The D1/D2 domain of the 26S subunit ribosomal DNA was also two-way sequenced for all the isolates obtained from experimental treatments.

(b) *Data analyses*

DNA sequences of yeast isolates were used to assess the composition and diversity of yeast communities found in the natural nectar samples of *H. foetidus* and in the artificial assemblages generated by the bee-only and bee + nectar experimental treatments. The nucleotide collection databases at GenBank were queried with the Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1997) to look for described yeast species with DNA sequences matching those of our isolates. All sequences queried ($n = 136$) yielded highly significant alignments with named yeast accessions in GenBank databases, most often at very high levels of sequence identity and coverage (99–100%). In a few cases (5.2% of queries, all from experimental groups), sequence identity was slightly lower (96–98%), which might denote the presence of undescribed taxa or species without accessions in the GenBank databases. To evaluate possible biases in richness estimates caused by the presence of undescribed species, we also considered operational taxonomic units (OTUs) defined on the basis of similarity of DNA sequences (Hughes *et al.* 2001; Fierer *et al.* 2007; Shaw *et al.* 2008). Determination of the number of distinct OTUs occurring in a set of DNA sequences, and assignment of sequences to OTUs, was done with the program DOTUR (Schloss & Handelsman 2005), using a DNA dissimilarity cut-off of 3 per cent. Although this cut-off is more conservative than the 1 per cent threshold suggested for species-level rDNA differentiation in yeasts (Kurtzman & Robnett 1998), it is the threshold commonly used to distinguish 'molecular' species of micro-organisms in recent environmental studies (Fierer *et al.* 2007; Peay *et al.* 2008).

Although both the number of bumble-bees assayed in the experiments and the number yielding some yeast colony were similar for the bee-only ($n = 30$ and 22 bumble-bees, respectively) and bee + nectar treatments ($n = 30$ and 20, respectively), the number of yeast isolates obtained differed between treatments, hence rarefaction methods were applied to compare diversities (Gotelli & Colwell 2001). Expected yeast species richness was estimated using individual-based rarefaction curves and the Chao2 non-parametric asymptotic estimator, treating each DNA sequence as a separate individual (Hughes *et al.* 2001). Expected richness and standard errors were computed with the program EstimateS (Colwell 2005).

The phylogenetic bias in the assembly of nectar yeast communities was examined by testing (i) whether isolates from the bee-only and bee + nectar treatments are unequally distributed over the common phylogenetic tree for all isolates combined, i.e. the two experimental groups harbour different lineages, and (ii) whether yeasts in the bee + nectar treatment represent a phylogenetically structured subset of those in the bee-only one, i.e. the nectar yeast community is phylogenetically clustered. The whole set of DNA sequences from the two experimental groups ($n = 97$) was aligned with ClustalW, and Gblocks was then used to eliminate poorly aligned positions and divergent regions (Castresana 2000). A phylogenetic tree was constructed for all isolates using MrBayes 3.1 (Ronquist & Huelsenbeck 2003), assuming a general time-reversible model and gamma-distributed rates. The hypothesis that the bee-only and bee + nectar communities harbour different lineages was tested with the parsimony test implemented in the program TreeClimber (Schloss & Handelsman 2006*a*). Given the phylogenetic tree constructed for all isolates, each tip was associated with one experimental group and the minimum number of transitions between groups was obtained using Fitch's (1971) algorithm. Statistical significance was tested by comparing that value with a null-model distribution obtained from randomly distributed experimental groups on the same phylogenetic tree. The hypothesis of phylogenetic clustering was tested with the 'comstruct' function of the Phylocom program (Webb *et al.* 2008). Net relatedness index (NRI), a standardized form of mean phylogenetic distance, was computed for yeast communities in the two experimental groups, and their significance assessed by comparison with randomly generated samples. Results obtained with the four null models available in the program were similar, and only those from the 'phylogeny shuffle' method will be presented.

3. RESULTS

Nectar samples from *H. foetidus* flowers exposed to natural pollinator visitation contained yeasts quite frequently. Yeasts were present in 72.5 per cent of the 80 nectar samples from the Las Navillas site that were examined microscopically. In yeast-containing samples, cell density ranged between 34 and 127 622 yeast cells per mm^3 (mean $\pm$ s.e. = 17 821 $\pm$ 2844 cells per mm^3, $n = 58$). A total of 39 isolates were sequenced, all of which belonged to a single species, *Metschnikowia reukaufii*.

Table 1. Taxon richness and species composition of the two groups of experimentally assembled nectar yeast communities considered in this study. The bee-only and bee + nectar experimental groups refer, respectively, to assemblages obtained by rubbing the glossae of bumble-bees on culture media and from probing *H. foetidus* virgin nectar with the bees' glossae, incubating for 48 h and then streaking the inoculated nectar on culture media.

	bee-only	bee + nectar
yeast isolates sequenced	37	60
observed number of taxa:		
named species, BLAST search	9	4
OTUs, 3% DNA dissimilarity cut-off	10	6
BLAST-identified, named species (number of sequences):		
Metschnikowia reukaufii	18	45
M. gruessii	3	11
Candida bombi	7	3
Kluyveromyces dobzhanskii	3	0
Hanseniaspora sp.[a]	2	1
H. osmophila	1	0
Saccharomyces bayanus	1	0
Cryptococcus saitoi	1	0
Candida friedrichii	1	0

[a]GenBank accession EF653942.1.

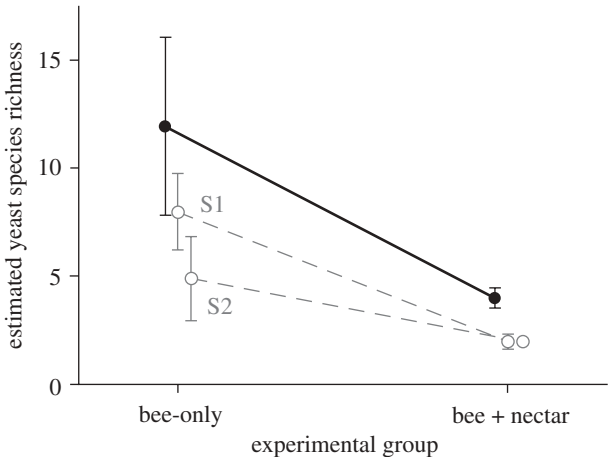

Figure 1. Estimated yeast species richness (Chao2 non-parametric estimator) for bee-only and bee + nectar experimental communities (black dots and continuous line; vertical segments denote ±1 s.e.). Bee-only and bee + nectar yeast assemblages refer, respectively, to those resulting from rubbing the glossae of bumble-bees on culture media, and from probing virgin *H. foetidus* nectar with the bees' glossae, incubating for 48 h, and then streaking the inoculated nectar on culture media. Grey dots and dashed lines depict species richness estimates computed separately for data subsets corresponding to bumble-bees captured at flowers of *H. foetidus* (S1) and *R. officinalis* (S2).

Per cent identity scores between their DNA sequences and the best alignments from BLAST queries were very high, ranging between 98 and 100 per cent (mean ± s.e. = 99.5 ± 0.1%).

A total of nine different yeast species were represented in the isolates obtained from the bee-only experimental treatment, belonging to the genera *Metschnikowia* (two species), *Candida* (two), *Hanseniaspora* (two), *Kluyveromyces* (one), *Saccharomyces* (one) and *Cryptococcus* (one) (table 1). Isolates from the bee + nectar treatment had lower observed species richness. Only four species were identified, representing a subset of those found in the bee-only treatment (table 1). The DOTUR-based analysis of DNA sequence data yielded similar results. A total of 10 OTUs were recognized in the bee-only isolates, while only six OTUs were identified in the bee + nectar isolates. Given the similarity between richness estimates based on named species and OTUs, we will consider hereafter only the results obtained using the first method.

The Chao2 non-parametric estimator of species richness reached (bee + nectar) or closely approached (bee-only) a plateau for the total number of isolates examined for each experimental group (results not shown). This indicates that the number of isolates sampled for each group was sufficient to provide reliable estimates of the expected total species richness. Chao2 richness estimates (±s.e.) were higher for the bee-only (11.9 ± 4.1 species) than for the bee + nectar group (4 ± 0.4 species), and the sign of the difference was robust to splitting the sample into subsamples consisting of the bumble-bees captured at *H. foetidus* and *R. officinalis* flowers (figure 1). In addition to differences in observed and estimated yeast species richnesses, the two experimental groups differed in evenness, with the most abundant species (*M. reukaufii*) accounting for 48.6 and 75 per cent of isolates in the bee-only and bee + nectar groups, respectively (table 1).

The phylogenetic tree constructed for the combined set of 97 isolates from the two experimental groups is shown in the electronic supplementary material, appendix. Yeast isolates associated with the bee-only and bee + nectar experimental groups harboured different lineages, as denoted by the highly significant result of the parsimony test ($p = 0.0006$). In addition, bee + nectar samples were clustered relative to random communities of equal richness assembled from the pool of all yeasts found in bee-only samples (i.e. the pool of all yeast species in all samples), as revealed by their NRI departing significantly from null model expectations (table 2). Results were nearly identical when separate analyses were conducted on data from bumble-bees collected from *H. foetidus* and *R. officinalis* (table 2). Taken together, these results show that yeasts communities experimentally assembled in the nectar comprised a phylogenetically biased, clustered subset of isolates occurring on bumble-bee glossae, as illustrated in the simplified, species-level phylogenetic tree shown in figure 2. With the only exception of a single isolate of *Hanseniaspora* sp., all isolates from the bee + nectar group clustered into a distinct 'nectarivorous' lineage with 100 per cent Bayesian credibility comprising *M. reukaufii*, *M. gruessii* and *Candida bombi*.

4. DISCUSSION

Our inventories of yeast species in natural nectar samples of *H. foetidus* corroborate the results of previous studies showing that, at least in temperate habitats, culturable yeast communities associated with floral nectar are characterized by low species richness (Vörös-Felkai 1957; Eisikowitch *et al.* 1990; Brysch-Herzberg 2004).

Table 2. NRI (a standardized measurement of mean pairwise phylogenetic distance) for yeast isolates in the two groups of communities assembled experimentally. Subsets 1 and 2 refer to data from bumble-bees captured at flowers of *H. foetidus* and *R. officinalis*, respectively. *p*-Values were calculated by comparing the observed mean pairwise phylogenetic distance with a null distribution for 10^5 randomly generated phylogenies, obtained by shuffling yeast isolates across the tips of the phylogeny shown in the electronic supplementary material, appendix (phylogeny shuffle method in the Phylocom program). The *p*-values shown correspond to one-tailed tests, and stand for the probability of obtaining by chance alone a mean phylogenetic distance as extreme as the observed one. NRI significantly greater than 0 denote phylogenetic clustering.

	bee-only		bee + nectar	
dataset	NRI	*p*-value	NRI	*p*-value
whole sample	−4.26	<0.00001	+5.17	<0.00001
subset 1	−2.17	0.013	+6.54	<0.00001
subset 2	−2.18	0.013	+0.23	0.41

During the two-week sampling period, yeast communities in *H. foetidus* nectar from the Las Navillas site consisted of a single species, *M. reukaufii*. The experimental yeast assemblages obtained by inoculating nectar with yeasts on bumble-bee glossae were slightly more diverse, containing three additional species (*M. gruessii*, *C. bombi* and *Hanseniaspora* sp.). Since bumble-bees used in the experiments were collected over a broader area than natural nectar samples, the difference in yeast diversity between natural *H. foetidus* nectar and experimental bee + nectar samples probably reflect variation over the sampling area in the composition of yeast communities on bumble-bee glossae. Another question raised by our results concerns the origin of inocula of yeast species that were unable to grow in *H. foetidus*: if they are not found in *H. foetidus* nectar, then where did bumble-bee glossae get contaminated with inocula of these species? Their most likely origins are honey pots at the bumble-bees' nests, pollen and flower surfaces contacted during foraging bouts, and floral nectar of other plants (Lachance *et al.* 2001; Brysch-Herzberg 2004).

Both observed and estimated yeast species richness was lower in nectar (bee + nectar treatment) than on the bumble-bee glossae used to inoculate it (bee-only treatment), which demonstrates that *H. foetidus* nectar is filtering the multi-species set of inocula brought in by foraging bumble-bees. Circumstantial evidence suggests that such habitat filtering is most likely due to *H. foetidus* nectar representing a strongly inhibitory environment that constrains the growth of most yeast species, and that only a few nectar specialists possessing certain physiological abilities can successfully develop there. *Helleborus foetidus* nectar contains protoanemonin (R. Pérez, I. M. García & C. M. Herrera 2009, unpublished data), an unsaturated lactone that inhibits the growth of some yeasts even at extremely low concentrations (Mares 1987; Kyung *et al.* 2007; A. Canto 2009, unpublished data). In addition, sugar concentration of virgin *H. foetidus* nectar is often very high (mean ± s.e. = 40.5 ± 1.2% w/w, range = 28–58%, $n = 50$; C. M. Herrera 2009, unpublished data), as usual among bee-pollinated plants (Nicolson *et al.* 2007). The osmotic stress associated with these high sugar concentrations is probably a limiting environmental factor, since tolerance to high sugar concentrations is a specialized physiological trait (Tokuoka 1993) possessed by only a small fraction of yeast species (around 13%, according to data in Barnett *et al.* 2000). Consistent with our interpretation that osmotic stress is an ecological factor contributing to nectar filtering is the observation that the three main species unaffected by filtering (*M. reukaufii*, *M. gruessii*, *C. bombi*) can grow on culture media containing 50 per cent glucose, while at least three of the species filtered by nectar cannot (e.g. *Saccharomyces bayanus*, *Cryptococcus saitoi*, *Hanseniaspora osmophila*) (Barnett *et al.* 2000). In a comparative screening of 252 strains of micro-organisms, *M. reukaufii* exhibited the highest arabitol production rate when subjected to intense osmotic stress (Nozaki *et al.* 2003). Since arabitol is one of several osmolytes playing an essential role in osmoregulation by osmotolerant yeasts (Grant 2004), that finding supports the suggested association between the ability of a yeast species to tolerate high osmotic pressure and its capacity to go through the ecological filter set by nectar.

Since high sugar concentration and presence of secondary compounds are common features of floral nectars (Adler 2000; Nicolson *et al.* 2007; González-Teuber & Heil 2009), the low species diversity prevailing in nectar yeast communities so far studied could reflect a generalized environmental filtering similar to that documented here for *H. foetidus*. Very low nitrogen content, another characteristic feature of floral nectars (Nicolson *et al.* 2007), may be yet another factor limiting the suitability of floral nectars as habitats for yeasts other than highly specialized nectarivores. In this respect, it is interesting to note that the two species contributing most isolates in our study (*M. reukaufii* and *M. gruessii)* were also the most abundant ones in nectar samples from 143 insect-pollinated species from Central Europe, where they accounted for 73 per cent of all isolates (Brysch-Herzberg 2004). In a survey of the nectar yeasts of 22 species of southern Spanish plants, *M. reukaufii* and *M. gruessii* accounted altogether for 87 per cent of all isolates (M. I. Pozo 2009, unpublished data). Given the close phylogenetic relatedness of the two species (Hong *et al.* 2003), and the frequent association between phylogeny and physiology exhibited by yeasts (Middelhoven & Kurtzman 2003), the dominance of these two *Metschnikowia* species in western European nectar yeast communities is probably associated with their possessing some suite of physiological traits allowing them to overcome nectar filtering. A combination of osmotolerance, tolerance or resistance to secondary compounds and efficient nitrogen use possibly allows these specialists to exploit floral nectar.

Many studies have shown that patterns of co-occurrence of taxa can deviate from random expectations with regard to phylogenetic relatedness (Emerson & Gillespie 2008; Vamosi *et al.* 2009), but microbial communities have been infrequently examined in a phylogenetic context and the few studies available provide contrasting results. Anderson *et al.* (2004), working on yeast communities in decaying cactus tissues, found associations between yeast species abundance and phylogenetic relatedness to be variable and contingent on features of the

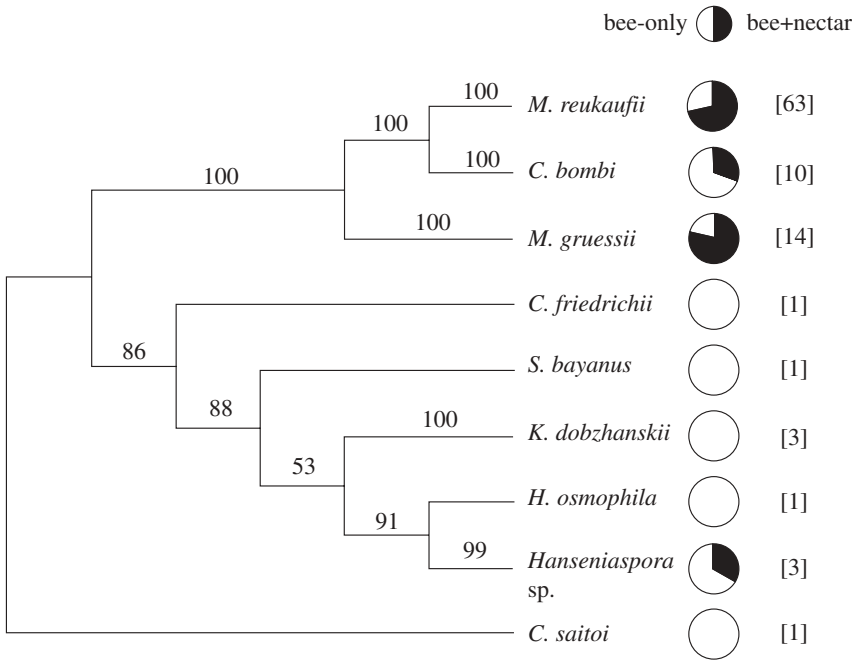

Figure 2. Phylogenetic tree summarizing the evolutionary relationships between the nine yeast species identified in this study, obtained by the Bayesian inference analysis of DNA sequence data from individual isolates. The simplified tree and Bayesian clade credibility (posterior probability) values presented here were generated by culling taxa from the larger phylogram depicting the relationships between the 97 isolates from the two experimental groups combined, shown in the electronic supplementary material, appendix. Clade credibility values, expressed as percentages, are shown next to the branches (are missing from branches represented by single isolates). Pie charts denote the proportion of isolates of each species in the bee-only (open sectors) and bee + nectar (filled sectors) experimental treatments. Total numbers of isolates per species are shown in brackets (see also table 1).

environment. In contrast, bacterial communities are often phylogenetically clustered, a pattern generally interpreted as denoting habitat filtering when closely related taxa share some phylogenetically conserved trait(s) that allows them to tolerate the abiotic conditions of a given habitat (Webb *et al.* 2002; Emerson & Gillespie 2008; Vamosi *et al.* 2009). The interpretation of phylogenetic community structure in natural systems, however, is subject to many confounding factors, and experimental manipulative investigations can be essential to unravel the underlying mechanisms involved (Maherali & Klironomos 2007; Vamosi *et al.* 2009). By directly assessing, rather than inferring, the species composition of the regional species pool (bumble-bee glossae) and experimentally ensuring that all species in the pool were equally likely to colonize the focal habitat (nectar), our study provides compelling support for the connection between habitat filtering and phylogenetic clustering, and also allows the identification of the likely ecological mechanism underlying clustering. As discussed above, the phylogenetically clustered subset of species present on bumble-bee glossae that were able to grow in *H. foetidus* nectar apparently share some capabilities to tolerate the harsh nectar environment, as assumed by the hypothesis linking habitat filtering and phylogenetic clustering (Vamosi *et al.* 2009).

Certain floral traits involved in mutualistic interactions with pollinators can be partly explained as the outcome of selection to reduce the impact of exploiters on plant fitness (Irwin *et al.* 2004). In this context, the so-called antimicrobial hypothesis interprets the toxic substances often occurring in floral nectars as defences against nectarivorous microbes (Adler 2000; González-Teuber & Heil 2009). One key assumption underlying the antimicrobial hypothesis, that the deleterious effects of microbes on nectar can be sufficiently severe as to select for antimicrobial compounds, was recently supported by studies on southern Spanish and South African plants showing that yeasts induce substantial degradation of floral nectar (Herrera *et al.* 2008; de Vega *et al.* 2009). The present investigation adds support for another central assumption of the antimicrobial hypothesis, that nectars with secondary compounds are protected from nectarivorous microorganisms. Toxic substances in *H. foetidus* nectar, however, do not confer protection against specialized microbial consumers (see also Manson *et al.* 2007), just as allelochemicals in other plant parts do not defend them from specialized herbivores (Bowers & Puttick 1988). Poisons in *H. foetidus* nectar possibly contribute to filter out unspecialized yeasts in the same way as plant allelochemicals filter out unspecialized herbivores. Under this hypothesis, the specialized nectarivores *M. reukaufii*, *M. gruessii* and *C. bombi* are predicted to tolerate protoanemonin and perhaps other inhibitory substances commonly found in nectar, just like specialist herbivores are immune to the allelochemicals of their host plants. This parallelism, if substantiated by other studies, would suggest an appealing similarity of the defensive mechanisms evolved by plants against microbial and non-microbial antagonists.

We are grateful to Pedro A. Tíscar and the Centro de Capacitación y Experimentación Forestal de Vadillo-Castril, Cazorla for essential laboratory space and facilities; José L. Garrido for advice on phylogenetic methods; André

Lachance for useful advice; Clara de Vega for discussion and comments on the manuscript; and Lawrence Harder and two anonymous reviewers for suggestions on the manuscript. Permission to work in the Sierra de Cazorla was granted by the Consejería de Medio Ambiente, Junta de Andalucía. Funding for this work was provided by grants P06-RNM-01627 (Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía), CGL2006-01355 and EXPLORA CGL2007-28866-E/BOS (Ministerio de Educación y Ciencia, Gobierno de España) and FPI Predoctoral Fellowship BES-2007-17142 from Ministerio de Educación y Ciencia to M.I.P.

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