RESEARCH ARTICLE

Nectar yeasts of two southern Spanish plants: the roles of immigration and physiological traits in community assembly

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Abstract

Recent studies have shown that dense yeast populations often occurring in floral nectar are numerically dominated by a few species from the flower–insect interface specialized genus *Metschnikowia*, while generalist yeast species commonly occurring on leaf surfaces, soil, freshwater, and air were rarely isolated from nectar samples. This study was designed to understand the main factors responsible for the assembly of nectar yeast communities, by combining field experiments with laboratory tests characterizing the physiological abilities of all yeast species forming the pool of potential colonizers for two Spanish flowering plants (*Digitalis obscura* and *Atropa baetica*). Yeast frequency and species richness were assessed in external sources (bee glossae, air, plant phylloplane) as well as in pollinator rewards (pollen, nectar). Yeasts were most frequent in external sources (air, flower-visiting insects), less so in the proximate floral environment (phylloplane), and least in pollen and nectar. Nectar communities appeared to be considerably impoverished versions of those in insect glossae and phylloplane. Nectar, pollen, and insect yeast assemblages differed in physiological characteristics from those in other substrates. Nectarivorous *Metschnikowia* were not more resistant than other yeast species to plant secondary compounds and high sugar concentrations typical of nectar, but their higher growth rates may be decisive for their dominance in ephemeral nectar communities.

Introduction

Microbial community dynamics in plants and on plant surfaces have been studied for over a century (e.g. Ruinen, 1963; Last & Price, 1969; Phaff & Starmer, 1987; Thompson *et al.*, 1993; Lindow & Brandl, 2003; Sampaio *et al.*, 2007), but many ecological processes that affect the microbial diversity still remain to be elucidated (Kinkel, 1997). Individual plants provide a multitude of microhabitats with different topographical features, nutrients, water availability, and a range of microclimatic conditions that select for diverse microbial communities (Andrews & Harris, 2000). Some yeast microhabitats within plants have been described to be specific. These include decaying tissues, flowers, nectar, or fruits (Phaff & Starmer, 1987; Lachance *et al.*, 1989; Starmer *et al.*, 1990; Rosa *et al.*, 1992, 1994; Spencer *et al.*, 1992).

In some studies carried out recently in south-eastern Spain, yeast communities of nectar were shown to possess high population densities and a clear predominance of members of the genus *Metschnikowia* (Herrera *et al.*, 2009, 2010; Pozo *et al.*, 2011). The few additional yeasts found in nectar consisted of generalist species that are known to occur in other microenvironments such as leaf surfaces, soil, freshwater, and air (Herzberg *et al.*, 2002; Brysch-Herzberg, 2004).

Microbial population dynamics in floral nectar, as in any other habitat, may be considered a complex function of four processes: immigration, emigration, growth, and death (Fonseca & Inácio, 2006). It is therefore reasonable to ask whether floral nectar is depleted at the same rate as the overall incoming yeast community or whether it represents a set of identical individual microhabitats that constrain colonization to a small group of highly specialized

yeast species. In comparison with other plant parts, spring or summer flowers have a very short lifespan. Inside the flowers, nectar is a highly fluctuating habitat that experiences evaporation at high temperatures, dilution by rain, and depletion from pollinator visits. Nectar colonization by yeasts is constrained by their close association with the flower-visiting insects, which both introduce the yeasts through their mouthparts and consume them along with the nectar (Gilbert, 1980; Sandhu & Waraich, 1985; Brysch-Herzberg, 2004; Canto *et al.*, 2008). The complex interaction associated with colonization history is compounded further with yeast survival and growth processes. On the one hand, the high sugar content common to floral nectars (Nicolson & Thornburg, 2007) may serve as a source of nutrients that can support microbial development and at the same time may render the habitat selective in favour of highly osmotolerant yeasts. Furthermore, nectarivorous diets are also characterized by a low nitrogen content (Nicolson, 2007). On the other hand, nectars of many plant species contain plant secondary compounds (Adler, 2000; Manson *et al.*, 2007; Nicolson, 2007; González-Teuber & Heil, 2009) that are thought to act as a deterrent for nectar robbers (e.g. inefficient pollinator insects, nectar-consuming microbial communities), thus favouring 'legitimate' visitors. In this context, yeasts deplete nectar sugars, devaluate the floral reward (Herrera *et al.*, 2008), and consequently might be affected by the presence of these substances. In plant species that produce defensive compounds, as is the case with those used in this study, these compounds may limit the number of yeast species that finally constitute nectar communities. The failure of microorganisms to survive in floral nectar may then be due to nectar physical conditions themselves, as it has been noted above, or to competition processes. Given that floral nectar is both an overpopulated (Herrera *et al.*, 2009) and a resource-limited habitat, the competitive advantage of *Metschnikowia* species may also be the result of a superior rate of reproduction and survival, which in turn leads to a reduction in competitors.

The main objective of this study was to better understand the origin and composition of the yeast species pool potentially arriving into floral nectar in two different plant species at the plant population scale. To this end, several microhabitats are evaluated as potential sources of yeast to floral nectar, and yeast frequencies are measured at different levels following a hierarchical sampling scheme in each individual plant. The levels range from external sources such as flower-visiting bee glossae, air, bracts, and corolla surfaces to floral rewards such as nectar and pollen. Measures of yeast species transference to floral nectar are indicative of the degree of microsite specialization, which raises the question of the mechanisms underlying nectar yeast community assemblage. Accordingly, this study examined whether interspecific differences in carbon and nitrogen sources utilization patterns, resistance to inhibitors and plant defensive compounds, osmotolerance, and growth rate contribute also to explaining features of the nectar yeast community.

Materials and methods

Field sampling

A total of 234 yeast isolates were obtained from the two study species, willow-leaved foxglove (*Digitalis obscura*, Plantaginaceae; *Digitalis* hereafter) and *Atropa baetica* (Solanaceae; *Atropa* hereafter), in 2009, in the Sierra de Cazorla region, a well-preserved natural area in Jaén Province, south-eastern Spain. Each plant species contributed one population, and the two were sampled sequentially according to their blooming period, which ranges from early June to late July. Samples were collected from each locality within 4 consecutive days. The *Digitalis* sampling site contributed 162 yeast isolates, and the other 72 were recovered from *Atropa*. The two localities were located 7.9 km apart.

Yeast isolates were obtained in a spatially nested scheme that ranged from external sources such as bees' glossae and air samples to plant-related samples obtained from 20 randomly selected plant individuals in each sampling site. Two flowers were sampled from each individual, from each of which one subsample was systematically collected from bracts, corolla outer and inner surfaces, pollen, and nectar samples. To avoid artefacts caused by different yeast colonization times, mature flowers that were 3 days old were systematically collected. *Digitalis* plants produce protandrous flowers that last for up to 5 days. In contrast, flowers of *Atropa* are markedly protogynous and last for up to 3–4 days. Nectar production at both species tend to be higher at the intermediate phase of floral development, which coincided with the female phase in *Digitalis* and the male phase in *Atropa* flowers (M.I.Pozo, pers. obs.).

Three different treatments were performed in this study. The first consisted of 10 individual flowers naturally exposed to insect visitation in each sampling site, and the second consisted of flowers of 10 other plants excluded from pollinator visitation by bagging at the bud stage ($N = 20$ flowers in each treatment; 'exposed' and 'insect-visitors-excluded', hereafter). After completing *Digitalis* experiments, it was detected that nectar from insect-visitors-excluded plants still harboured yeast in a significant fraction, and so two additional buds in each of the ten previously bagged plants were covered with

cellophane envelopes ('airflow-prevented' samples, $N = 20$ flowers).

Eighteen and 15 bees were hand-netted while they foraged on *Digitalis* and *Atropa* sampling sites, respectively. Three *Bombus* species were collected from *Digitalis* plants (*Bombus pascuorum*, *Bombus pratorum*, and *Bombus terrestris*), comprising both workers and males; *B. terrestris* (males), *B. pascuorum* (workers), and the solitary bees *Anthidium florentinum* and *Anthophora* (*Amegilla*) *quadrifasciata* were recorded in *Atropa* flowers. Based on pollination censuses carried out simultaneously for both sampling sites (see Herrera *et al.*, 2001 for censuses methodology), both bee pools were a representative sample of the whole set of visitors (Supporting Information, Table S1). Immediately upon capture, bees were placed individually in sterile containers and anaesthetized by placing them for 2 min inside a freezer at −20 °C. The glossa of each individual bee was carefully extended, using sterile forceps, beyond the tip of the maxillary galeae and carefully rubbed against the surface of an agar plate. Airborne yeast samples were obtained by placing an open agar plate under each selected plant for 5 min.

Entire flowers and their most proximate bract were collected in the field and kept refrigerated in a sterile container until they were brought to the laboratory. The bracts and corollas were gently swabbed for inoculation on agar medium. Inner and outer surfaces of the corollas were also sampled by imprinting them onto agar medium. Pollen was sampled by immersing a mature anther in 1 mL of sterile water for 48 h and streak-inoculating 1 μL of this inoculum onto an agar plate. Nectar samples were taken with 1-μL calibrated microcapillaries and transferred onto agar plates for streak inoculation.

Yeast isolation, identification, and physiological characterization

All samples were cultured onto yeast malt agar plates (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, and 2.0% agar) with 0.01% chloramphenicol and incubated at 25 °C.

Isolates were purified and characterized following the standard methods of Yarrow (1998). Growth responses were evaluated by replica plating onto 115 test media developed by Lachance (1987) or specifically conceived for this study. This included growth on 44 carbon sources, 20 inhibitors, five nitrogen sources, four fermentation tests, three tests of hydrolysis, three halotolerance tests, three tests of morphological characteristics, two carbon and nitrogen sources tests, two growth factors, and individual tests for osmotolerance, acid production, colour reaction, and starch production, and 25 growth temperatures. The D1/D2 domains of the large subunit ribosomal RNA gene were two-way-sequenced for all the isolates as described in the study of Lachance *et al.* (1999), using the primer pair NL1/NL4. Gblocks (Castresana, 2000) was used to trim the resulting alignment, and the DNA sequences were compared with those in the GenBank database (last accessed 9 June 2010) by querying with the BLAST tool. Using the same procedure as Pozo *et al.* (2011), operational taxonomic units (OTUs) were also evaluated, defined on the basis of DNA sequence similarity with the program DOTUR using the 3% similarity threshold (distance-based OTU and richness, Schloss & Handelsman, 2005).

Plant secondary compounds assays

Agar diffusion was used to evaluate the possible effect of plant defensive compounds present in nectar on yeast growth. Three of the major compounds in each plant were tested, namely the tropane alkaloids atropine, tropine, and scopolamine from *Atropa* (Zárate *et al.*, 1997; Adler, 2000), and the digitoxose-type cardenolides digitoxin, digitoxigenin, and gitoxigenin from *Digitalis* (Gavidia & Pérez-Bermúdez, 1997; C.M. Herrera, unpublished data). The potential effect of these compounds was assessed on 12 yeast strains, each from a different species: *Candida bombi*, *Candida floricola*, *Cryptococcus laurentii*, *Cryptococcus victoriae*, *Debaryomyces maramus*, *Lachancea thermotolerans*, *Metschnikowia reukaufii, Metschnikowia gruessii*, *Metschnikowia kunwiensis*, *Rhodotorula colostri*, *Starmerella bombicola*, and *Sporobolomyces roseus*. Plates of Yeast Nitrogen Base (Difco) plus 1% of glucose and 1.5% agar were inoculated with dilute yeast suspensions of each yeast strain, and 5 μL of dimethyl sulfoxide (DMSO) solutions containing 1 mg of each compound was added as individual drops. Lack of toxicity of DMSO was corroborated for yeast strains by exposing yeasts to 5 μL of this solvent. The plates were incubated at 24 °C and examined periodically for the evidence of inhibition zones induced by secondary compounds.

Osmotolerance and optimal growth temperature assays

The growth responses to increasing proportions of glucose in agar media was tested for 144 strains belonging to the 29 yeast species recovered from the different microhabitats studied. The media contained 1% yeast extract, 1.5% agar, and 40, 45, 50, or 55% (w/w) glucose, and the plates were incubated at 24 °C. Growth of the same strains was evaluated on Yeast Malt (YM) agar at four, six, eight, 10, and 12 °C and from 26 to 42 °C with increments of one degree. Yeast growth was recorded on an ordinal scale after three, seven, and 18 days. For taxa represented by more than one specimen, the most common response is reported.

Data analyses

Sample-based occurrence data were used to obtain observed species richness (Mao Tau function, Mao *et al.*, 2005) and to calculate species richness estimators (ICE and Chao2) with ESTIMATES version 8.2 (Colwell, 2005). This program was used also to conduct analyses of shared species between microhabitats using sample-based abundance data. The Chao-shared estimator was used to compare species co-occurrence between microhabitats. For these analyses, bee glossae and air samples were considered together as 'external sources' samples, and bracts and the two corolla surfaces as 'phylloplane' samples. Pollen and nectar isolates were combined as 'pollinator rewards' samples. For the purpose of analysing the physiological features of the yeast isolates, an 82-test subset from the replica-plating series was selected by choosing those tests for which more than 5% of the strains differed in growth response. This subset comprised 39 carbon sources, 16 temperatures, 12 inhibitors, five nitrogen sources, three halotolerance and one osmotolerance tests, three tests of lytic activity, one test of carbon and nitrogen source, and individual tests for vitamin independence and glucose fermentation. The growth responses for these tests for 50 yeast strains were analysed using principal component analysis (PCA) with cross-validation on the covariance matrix (STATISTICA 7.0; StatSoft). Interpretation of the data was made by inspection of the scores and loading plots for the first two components, which accounted for 41% of the total variance.

Twenty-nine yeast species were clustered according to growth responses to increasing sugar concentrations by calculating the Euclidean distance matrix as implemented in PCORD version 4.0 Clustering Analysis. An UPGMA tree was computed using PHYLIP Neighbour on this distance matrix.

In view of the temperature range to which yeasts were naturally exposed (C.M. Herrera, unpublished data) at both sampling sites during the sampling period, yeast growth slopes were represented from eight (minimum field temperature during experiments) to 30° (maximum). Although yeast growth was followed over 18 days, considering the ephemeral nature of flowers as yeast habitats and, more specifically, that flowers of *Digitalis* and *Atropa* last up 4–5 days (M.I. Pozo, unpublished data), only data on growth rate within 3 days of incubation at each temperature were included in the analyses.

Results

Occurrence of yeast species in different microhabitat types

The frequency of samples containing yeasts was the highest in external sources such as air samples and bees (above 70%), less so in samples belonging to the proximate environment of the flower, and the lowest in nectar and pollen samples, even though the two plant species studied differed in yeast frequency at each microsite (Fig. 1).

In insect-visitors–excluded plants, phylloplane samples continued carrying roughly the same proportion of yeasts, but pollen samples harboured yeasts at a 50% lower frequency (Fig. 2). The two plant species showed different patterns with respect to nectar yeast frequency in excluded and exposed treatments. Exposed *Digitalis* nectar contained yeasts in 60% of samples, this figure falling to 30% when insect visits were prevented. Nevertheless, in *Atropa* nectar, yeasts were only recovered in bagged flowers. When both airborne microbial contamination and pollinator visits were prevented at the same time, no yeasts were recovered from pollen and nectar samples, and corolla surfaces carried yeast in < 15% of samples.

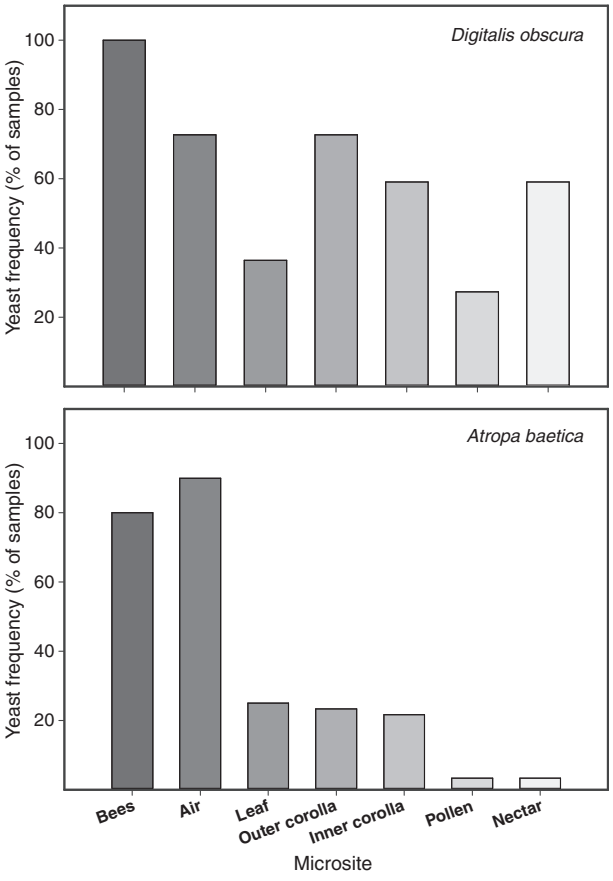

Fig. 1. Frequency of samples containing yeasts in each microhabitat for the two sampling sites studied.

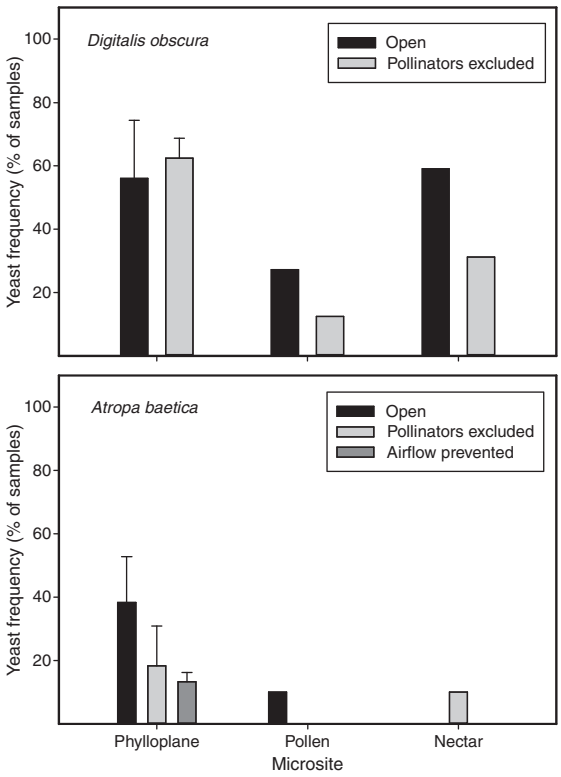

Fig. 2. Frequency of samples containing yeasts in the phylloplane (both corolla surfaces and bract mean ± SD), pollen, and nectar samples from the two plant species in sequential bagging experiments: exclusion of pollinator-vectored yeasts (upper panel) and airborne plus pollinator-vectored yeasts (bottom panel).

Spatial distribution of yeast species within plants

The 234 yeast isolates obtained at the two study sites belonged to 17 genera and 36 species (Table 1). Both plant species were characterized by similar amounts of yeast species richness, with 22 (*Digitalis*) and 23 (*Atropa*) species, respectively, in total. The DOTUR-based analysis of DNA sequences yielded fairly similar results in *Atropa* sampling site, with a total of 26 OTUs. In *Digitalis*, the method recognized up to 38 distinct OTUs, which suggests that some undescribed species may occur in the samples.

The yeast assemblages associated with the two plants were similar in showing a marked numerical dominance of a few species, namely the ubiquitous phylloplane species *Aureobasidium pullulans* plus *M. gruessii* that was recovered from all microhabitats except the air (Table 1). *Metschnikowia reukaufii* was frequently recovered from bees, and *Cryptococcus* species dominated the air and the phylloplane (Fig. 3).

Species richness did not follow the same pattern as did the yeast frequencies in samples. Bee glossae harboured eight and five yeast species in *Digitalis* and *Atropa*, respectively, and five yeast species were recovered from air samples at both sites. The highest yeast species richness was found in corolla samples and the lowest was in pollen and nectar, with two species in each substrate at both sites. Rarefaction analyses showed that the coverage of yeast species could be generally improved by additional sampling, except for *Atropa* local phylloplane and *Digitalis* nectar, whose curves appear to have reached a plateau with the existing sampling effort (Fig. 3).

Comparative composition of yeast assemblages in plants, insects, and air samples

Yeast communities isolated from corolla surfaces at the two localities were remarkably similar, judging by the total number of the Chao-shared estimated species (7). In addition, the yeasts of bracts and bee glossae local sets would share up to two yeast species when comparing the two sampling sites.

When considering the two sampling sites separately, the presence of shared species was also used to study patterns at yeast species' degree of interchange between microsites. The two plant species were characterized by different patterns of species similarity between microsites. In *Digitalis*, bee glossae and phylloplane samples shared up to eight yeast species. Some pollen yeast species would be shared (up to two) with phylloplane, bee glossae, and nectar samples. In *Atropa*, phylloplane and air samples were highly similar in species composition, with 31 shared species. In this sampling site, bee visitors would exchange a low proportion of species with phylloplane (1–2).

Microsites and physiological profiles

The relationship between physiological profile and microsite of origin of yeast isolates was explored by PCA. The first two axes accounted for 41% of the total variation. The main factors underlying the first component (24% of variance, PC1, Fig. 4) were growth on L-arabinose, 2-ketogluconate, inositol, raffinose, and gluconate ($r > 0.7$). The second component (PC2, 17% of total variance) was correlated with nitrate, nitrite, and phenylalanine ($r > 0.4$) as nitrogen sources and was moderately correlated with lipid hydrolysis and growth on D-glucuronate (see Table S2 in Supporting Information for further information). Yeast species did not show a well-defined clustering in a two-dimensional space defined by the first two axes of the PCA, but there is a microhabitat aggregation trend, in which those strains from air and phylloplane tend to disaggregate from those strains recovered from nectar, pollen, and bees' glossae.

Table 1. Species of filamentous fungi and yeasts recovered from air, bee glossae, phylloplane, nectar, and pollen samples, as a function of the plant species surveyed

	Digitalis obscura							*Atropa baetica*						
Fungal spp.	Bees	Air	Bract	Outer corolla	Inner corolla	Pollen	Nectar	Bees	Air	Bract	Outer corolla	Inner corolla	Pollen	Nectar
Aureobasidium pullulans	1	3	14	21	11	2	7	1	5	5	1	1		
Candida bombi				3								1		
Candida floricola								5						
Candida friedrichii					1								1	
Coniochaeta leucoplaca														1
Cryptococcus adeliensis		1												
Cryptococcus aerius		5		1		1								
Cryptococcus festucosus				1					1		1	1		
Cryptococcus laurentii	1													
Cryptococcus magnus											1	1		
Cryptococcus oeirensis			1							1	2	1		
Cryptococcus saitoi				1					1					
Cryptococcus stepposus									2			2		
Cryptococcus terreus		1												
Cryptococcus victoriae					1				3	2	2			
Debaryomyces hansenii					1									
Debaryomyces maramus	1			1	1						1			
Debaryomyces nepalensis								2						
Dothichiza pythiophila										1				
Dothiora elliptica											1			
Lachancea thermotolerans								1						
Metschnikowia gruessii	21		2	2	4	6	19							
Metschnikowia kunwiensis	1			2	4									
Metschnikowia reukaufii	11							12				1	1	
Ogataea zsoltii					1									
Rhizosphaera pini				1										
Rhodotorula mucilaginosa														1
Rhodotorula sp.		1												
Starmerella bombicola	1				2									
Sydowia polispora										1	1	1		
Trichosporon montevidensis												1		
Trichosporon moliniforme											1			
Ustilago maydis			1	1										
Ustilago sp.											2	1		
Zygosaccharomyces mellis	1													
Zygosaccharomyces rouxi											1			
Number of distinct species	8	5	4	10	9	3	2	5	5	5	11	10	2	2
Number of yeast isolates	38	11	18	34	26	9	26	21	12	10	14	11	2	2

Shaded boxes indicate the presence and the number of isolates of a species in a given sample type.

Osmotolerance and response to secondary compounds

No noticeable heterogeneity among yeast species was found in their resistance to six secondary compounds assayed in the subset examined. Cardenolides from *Digitalis* did not induce any inhibitory response irrespective of dose or strain tested. As to alkaloids, all the yeast species tested were equally susceptible to concentrations of atropine and tropine above 150 μg g^{-1}. Contrary to our initial hypothesis, *Metschnikowia* species did not exhibit a greater resistance to the toxic compounds assayed. In fact, only *Metschnikowia kunwensis* and *M. reukaufii* strains were sensitive to Scopolamine 150 μg g^{-1}.

Osmotolerance tests revealed that 16 of the 29 yeast species examined failed to grow at 40% glucose. A great gradation in yeast growth response under increasing glucose concentrations may be discerned for the subset of osmotolerant yeast species (Table 2). For example, *Zygosaccharomyces* and *Debaryomyces* were readily capable to grow at 55%. Other yeast species, such as *Starmerella* and *C. bombi* reached the same upper limit, but they showed

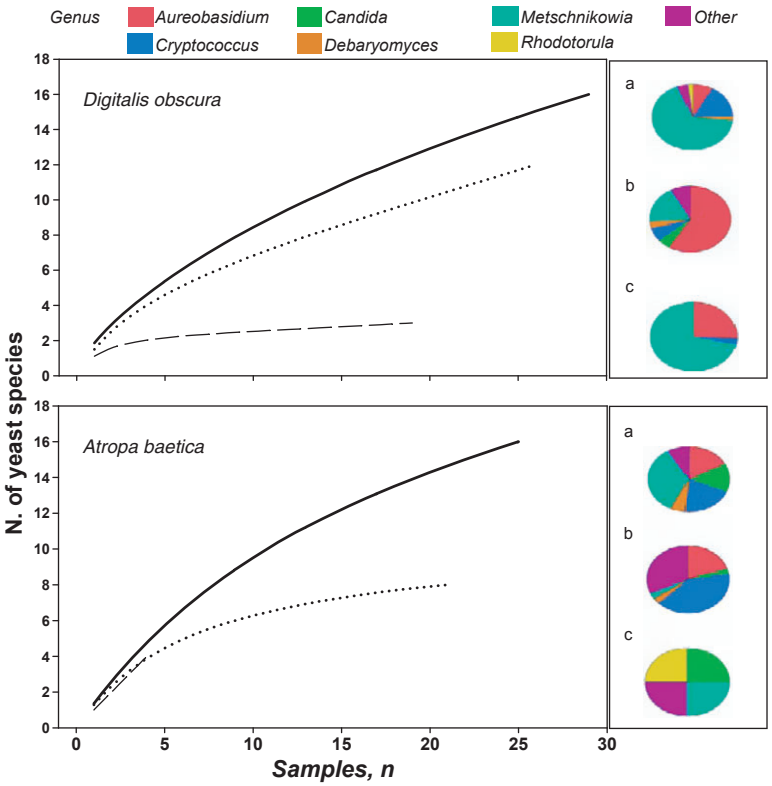

Fig. 3. Species accumulation curves (rarefaction analysis) separately by microhabitats in *Digitalis* and *Atropa* localities: external sources (solid lines), phylloplane (dotted lines), and pollen and nectar communities (long dash lines). Pie charts display the taxonomic distribution of isolates among genera at each microhabitat compartment (external sources, a; phylloplane, b; pollen and nectar, c).

a delayed growth response above 45–50% glucose. *Metschnikowia* strains mostly failed to grow at glucose concentrations above 50%. A classification of species based on their similarities in tolerance to high glucose concentrations did not reveal any distinct segregation of isolates from nectar and bee glossae (related to high glucose microenvironments) with respect to isolates from the phylloplane and the air (Table 2). However, highly osmotolerant yeast species such as *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Candida friedrichii*, and *C. bombi* were found only among phylloplane isolates, and two of the three nectar yeast species did not grow in 40% of glucose medium (Fig. S1).

Growth rates and temperature responses

As many as 38% of the species tested failed to grow during the first 3 days after plating, irrespective of temperature. Species with delayed growth responses comprised six *Cryptococcus* species (*C. festucosus*, *C. laurentii*, *C. magnus*, *C. oeirensis*, *C. stepposus*, and *C. terreus*), along with the yeast-like fungus *Coniochaeta leucoplaca* and the plant pathogens *Dothiora elliptica* and *Dothichiza pythiophila*. Only 17% of the yeast species grew at temperatures below 12 °C within 3 days (Fig. 5). Specifically, *D. hansenii* and *C. friedrichii* had minimum growth temperatures of 8 °C, and the three *Metschnikowia* species were the only isolates able to grow below this temperature. As to the upper limit of temperature tolerance, three species failed to grow at or above 30 °C (*C. friedrichii*, *C. victoriae*, and *D. nepalensis*). Only four species grow over the entire range observed in the field (8–30 °C). Those were *D. hansenii*, *M. gruessii*, *M. kunwiensis*, and *M. reukaufii*. In terms of cell density, five species reached the highest value of growth in this 3-day trial, namely *C. bombi*, *D. hansenii*, *M. reukaufii*, and *Ogataea zsoltii*.

Discussion

Despite a substantial body of literature on air- and insect-borne propagules and their significance to dispersal of epiphytic microorganisms (e.g. Andrews, 1991; Kinkel, 1997; Lachance *et al.*, 2001; Starmer *et al.*, 2003), there have been few attempts to correlate quantitatively shifts in air and insect yeast inocula with specific changes in

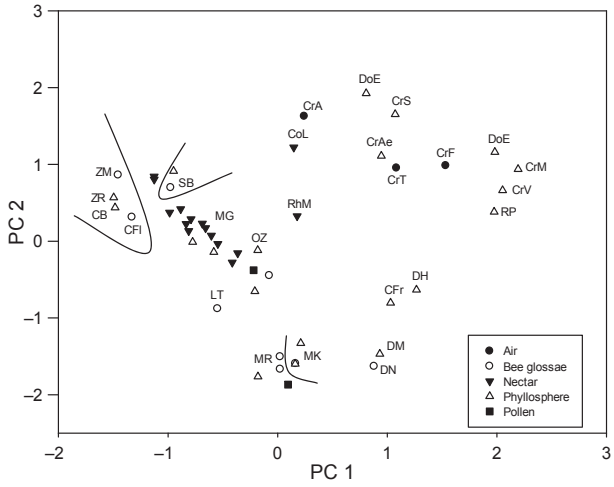

Fig. 4. Plot representing the principal components analysis of the physiological characteristics of 50 yeast strains and their natural microsite of origin. The first two principal components (PC1 and PC2) accounted for 41% the total variation observed. The fungal species represented here are *Candida bombi* (CB), *Candida floricola* (CFl), *Candida friedrichii* (CFr), *Coniochaeta leucoplaca* (CL), *Cryptococcus adeliensis* (CrA), *Cryptococcus aerius* (CrAe), *Cryptococcus festucosus* (CrF), *Cryptococcus magnus* (CrM), *Cryptococcus terreus* (CrT), *Cryptococcus victoriae* (CrV), *Debaryomyces hansenii* (DH), *Debaryomyces maramus* (DM), *Debaryomyces nepalensis* (DN), *Dothichiza pythiophila* (DoP), *Dothiora elliptica* (DoE), *Lachancea thermotolerans* (LT), *Metschnikowia gruessii* (MG), *Metschnikowia kunwiensis* (MK), *Metschnikowia reukaufii* (MR), *Rhodotorula mucilaginosa* (RhM), *Ogataea zsoltii* (OZ), *Starmerella bombicola* (SB), *Zygosaccharomyces mellis* (ZM), and *Zygosaccharomyces rouxii* (ZR). Curved lines are drawn on chart to separate yeast species when needed.

phylloplane and floral yeast communities (e.g. for air and phylloplane interchange, Andrews *et al.*, 1987). It was found that external sources, namely atmosphere and flower-visiting insects, were quantitatively important yeast donors to the phylloplane and flowers of the two plant species studied. Our results showed that nectar and pollen yeasts were mainly vectored by bees' mouthparts and only secondarily from the atmosphere.

The yeast assemblages recorded in this study could be divided into three main groups according to their microhabitats. Communities of mostly ascomycetous, osmotolerant species were associated with insect mouthparts (i.e. *Debaryomyces*, *Metschnikowia*, *Starmerella*, or *Zygosaccharomyces* spp.), basidiomycetous (*Cryptococcus* spp.) yeasts were isolated primarily from phylloplane and air samples, and floral nectar harboured highly restricted communities, represented by *M. gruessii* in *Digitalis* and by two fungal species in *Atropa*, composed by the basidiomycetous *Rhodotorula mucilaginosa* and the ascomycete yeast-like fungus *C. leucoplaca*.

Yeasts from natural environments may be classified according to their growth characteristics (Davenport, 1976). In this study, yeast species associated with insect visitors and their floral rewards could be discerned from generalistic species from the phylloplane and the air on the basis of their carbon and nitrogen source utilization patterns. This division was largely attributable to the abundance of basidiomycetous yeast species on plant surfaces and the predominance of ascomycetous species in bees and nectar. Consistent with this distinction, the yeast communities of phylloplane and bee mouthparts were

Table 2. Comparative evaluation of osmotolerance of 13 yeast species* based on their growth response† on media containing 40–55% glucose.

Fungal spp.	Association with high-sugar environment (bee and/or nectar)	Growth index with increasing glucose concentration (%w/w)			
		40%	45%	50%	55%
Debaryomyces nepalensis	B	5	5	5	5
Zygosaccharomyces mellis	B	5	5	5	4
Zygosaccharomyces rouxii		5	5	5	4
Candida bombi		5	5	3	3
Debaryomyces maramus	B	5	5	4	2
Metschnikowia kunwiensis	B	6	5	3	2
Candida floricola	B	5	5	5	0
Metschnikowia reukaufii	B	5	5	4	1
Starmerella bombicola	B	5	4	3	3
Lachancea thermotolerans	B	5	5	3	1
Metschnikowia gruessii	B + N	5	5	3	1
Candida friedrichii		5	4	3	1
Debaryomyces hansenii		5	4	3	1

*The present list is a subset of the 29 yeast species used in osmotolerance test. The rest ones, which are those not capable to grow in media containing 40% glucose, are listed in Fig. S1.

†Growth index: missing data, 0; absence of growth, 1; weak, 2–4; apparent growth, 5–6.

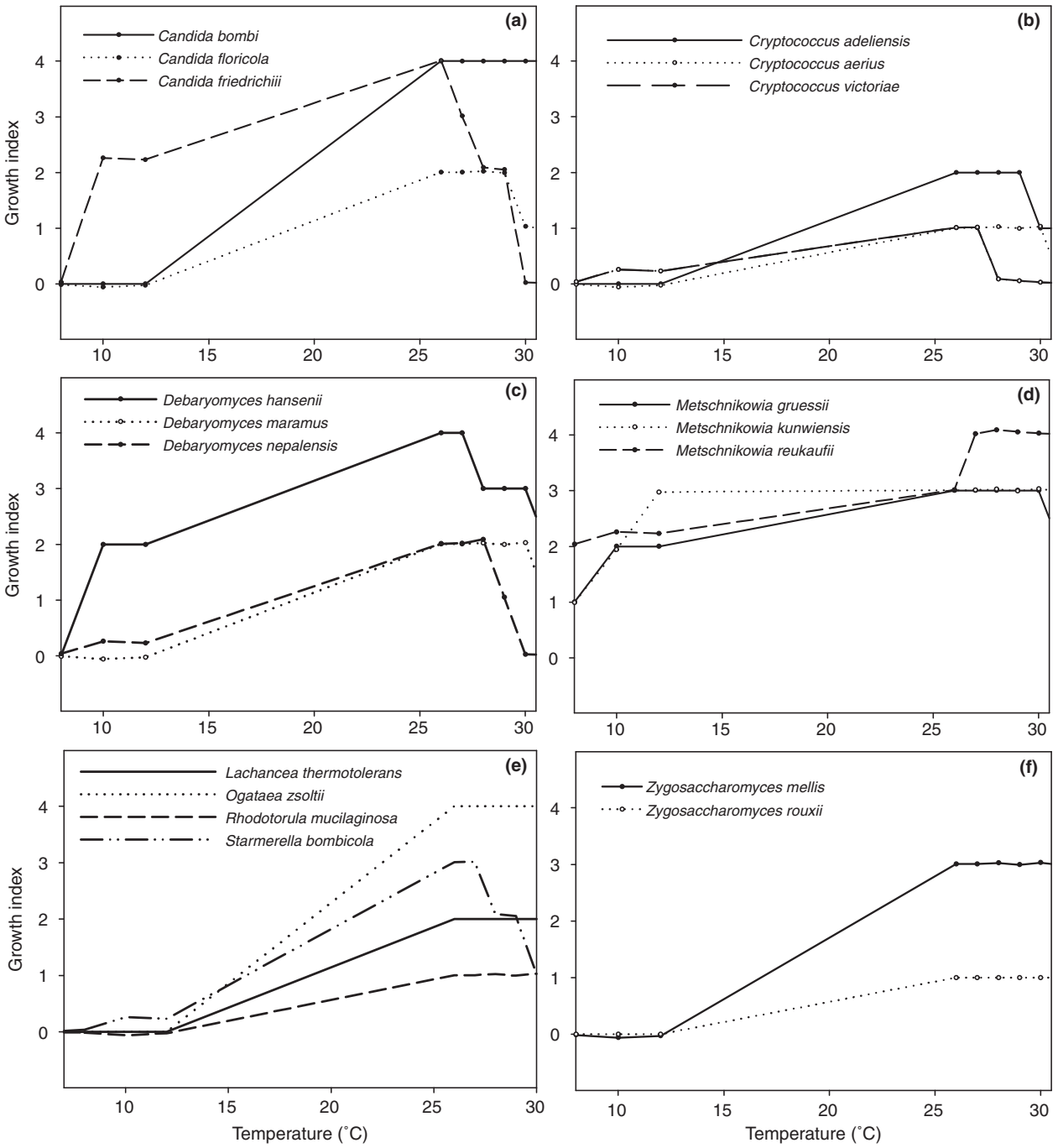

Fig. 5. Growth curves of 29 yeast species each belonging to the following genera: *Candida* (a), *Cryptococcus* (b), *Debaryomyces* (c), *Metschnikowia* (d), others (e), and *Zygosaccharomyces* (f) on YM medium at chamber temperatures from 8 to 30 °C. Growth index: absence of growth, 0; weak, 1; apparent growth, 2–4.

dissimilar. Typically, insect-vectored species, such as *Metschnikowia* spp., *Zygosaccharomyces* spp., and *S. bombicola* (Rosa & Lachance, 1998; Rosa *et al.*, 2003), have been also isolated at a lesser frequency from corolla surfaces, which might be interpreted as a sign of niche specialization, but this also indicates that phylloplane strains cover a very broad range of physiological abilities. The phylloplane is generally considered oligotrophic (Andrews & Harris, 2000), and depending on the system studied, carbon compounds alone or both carbon and

nitrogen compounds were shown to be limiting factors for bacterial and yeast populations on leaves (Bashi & Fokkema, 1977; Mercier & Lindow, 2000). From a microbe's perspective, the phylloplane is a continuously fluctuating physical environment, both spatially and temporally (Hirano & Upper, 2000; Lindow & Brandl, 2003), and so a high physiological plasticity at the community level may be considered an indispensable step for its colonization.

Typical phylloplane species were isolated in the nectar of *Atropa*. In this plant species, nectar samples harboured yeasts only when insect visits were excluded, and so it is not surprising that nectar and bee glossae did not share any species in this case. The fungal species appearing in nectar (*R. mucilaginosa* and *C. leucoplaca*) have been previously isolated as endophytes (Unterseher & Schnittler, 2010), suggesting that additional work should be done to assess the possibility that nectar yeasts in *Atropa* nectar may also originate from the plant tissues themselves. It is worth noting, however, that *R. mucilaginosa* is thought to be the most ubiquitous yeast known (Sampaio, 2010). Moreover, the two pigmented species found in *Atropa* nectar were isolated during summer months, when prevailing temperatures, moisture levels, day length, and intensity of sun exposure may be favouring those yeasts that presumably are better adapted: *C. leucoplaca* is a saprobic yeast-like fungus that is able to grow in dry conditions and whose maximum growth temperature is 34 °C (Cannon & Kirk, 2007, pp. 88), and *R. mucilaginosa* grows at temperatures up to 36 °C (data not shown).

The present study supports the conclusion that, in comparison with flower-visiting bees and phylloplane, nectar has a notably lower yeast frequency and harbours a very limited set of yeast species. The low species richness of nectar yeast communities provides support for the 'nectar-filtering hypothesis', as proposed in Herrera *et al.* (2010). According to this hypothesis, the high sugar content and the presence of plant secondary compounds that usually characterize nectar samples, as was noted in the introduction, may have a strong impact on the survival of incoming yeasts. The main nectarivorous *Metschnikowia* species, as described by this study and previous work conducted in the same region (Herrera *et al.*, 2009, 2010; Herrera & Pozo, 2010; Pozo *et al.*, 2011), may then possess some suite of specific physiological traits that allow them to successfully overcome nectar filtering.

Secondary compounds and sugars

The specific resistance of members of the Metschnikowiaceae clade to plant secondary compounds and high glucose concentrations will be discussed below, by comparing nectar-inhabiting species with the potential pool of species arriving to floral nectar in the two plant populations surveyed. In our attempt to detect specific resistance of nectar yeasts to toxic compounds produced by the plant species examined in this study, *Digistalis* cardenolides did not show any inhibitory effect on yeast growth, and *Atropa* alkaloids were equally toxic to all yeast colonizers. Given that the present assay tested concentrations of these compounds that go well beyond the proposed threshold for plant parts of *Atropa* sp. (Zárate *et al.*, 1997), the inhibitory effect of alkaloids may have been overestimated. It can be certainly concluded that nectarivorous yeast species did not show any advantage with respect to resistance to secondary compounds, as it was pointed out by Manson *et al.* (2007). Despite the lack of inhibitory effect of glycosides on yeast growth in our assays, it cannot be ruled out that a complex mixture of secondary compounds and primary metabolites, or even a mixture of these substances and inorganic ions, could be effective against yeasts. It is also possible that the commercial substances used in those experiments were slightly different from those occurring in nature. All the same, both plant species harboured yeasts in their nectar samples, such that it cannot be concluded that the plant secondary compounds used in our assay were inhibitors in natural conditions for nectar-depleting yeasts, as it has been proposed by the 'antimicrobial hypothesis' (Adler, 2000; Golonka, 2002, *as cited in* Antonovics, 2005; Irwin *et al.*, 2004; Manson *et al.*, 2007).

The principal factor thought to limit nectar community composition is the inability of a high proportion of colonizers to survive the high sugar conditions of nectar. *Atropa* nectar is characterized by a very high sugar concentration (per cent of total sugars, w/w, mean ± SD; 46.5 ± 22.1), and *Digitalis* nectar possesses more moderate sugar concentration (16.2 ± 2.9). In spite of this, most yeast species found to be osmophilic were not recovered from nectar samples from either of the two plant species surveyed. The exception was *M. gruessii*, which is able to grow in the presence of up to 50% glucose and was found in nectar samples examined in our study.

High sugar content and plant secondary compounds alone thus do not explain the prevalence of *Metschnikowia* species in floral nectar, which raises the question of what additional factors should be considered in the 'nectar-filtering' hypothesis. The possibility cannot be ruled out, however, that some of our negative results were the consequence of insufficient statistical power owing to relatively small sample sizes (i.e. committing type II error or failing to reject a false null hypothesis). The information available only allows to speculate that if such effects do actually exist, they probably are neither strong nor pervasive.

Recent studies have suggested that interspecific differences in thermal tolerance limits or growth responses can play a central role in yeast community organization by influencing the species composition of communities (Lachance *et al.*, 2003; Sweeney *et al.*, 2004; Goddard, 2008; Sampaio & Gonçalves, 2008). Differential thermal tolerance may determine what yeast species survive in natural populations for a given season, but this does not explain why the lowest species richness was in fact found in nectar samples. Moreover, draining and direct radiation may strengthen this effect on the phylloplane.

Yeast species growth rate

Rapid growth should be a significant advantage for nectar colonizers, given the ephemeral nature of this resource. Nectar secretion by individual flowers at the two spring–summer blooming plant species examined in this study lasts for up to 3 days. Yeast species with a delayed growth response, in such a short time span, should be 'swept' by competition from the faster-growing *Metschnikowia* species. Even at low temperatures, *Metschnikowia* species reach a high growth rate, higher than that of other species that is capable to grow between 8 and 31 °C within 3 days, such as *D. hansenii*. The temporal constraints imposed by the short duration of the floral microhabitat could also explain the lower frequency of yeasts in nectar samples compared to external sources and the phylloplane. Assuming that the ephemeral nature of flowers and/or the nectar secretion phase may be a strong limiting factor for nectar-colonizing yeasts, this duration-restricted effect may be strengthened in plant species with high visitation rates. In *Digitalis* plants, four species of social bees were the main visitors during our experiment. In the *Atropa* population, more insect visits were counted, and visitors covered a broader spectrum that included solitary bees, social bees, and Lepidoptera. When pollinator composition, nectar yeast frequency, and species richness were explored in a broader range of plant species and study sites in the same region (one population each of 13 plant species, M.I. Pozo and C.M. Herrera, unpublished results), the frequency of total flower visits by social bees was correlated positively with yeast frequencies in the nectar samples ($rs = 0.55$, $P = 0.05$). In contrast, the frequency of visits by solitary bees correlated positively with nectar yeast species richness (13 populations, $rs = 0.65$; $P = 0.01$).

In conclusion, results of this study strongly suggest a combined influence of immigration history and different ecological and selective forces operating at each microhabitat as the driving features ultimately determining yeast communities, in terms of both yeast incidence and yeast species composition. Additional work is needed to untangle the complex combination of suggested factors here, and the main conclusions of this study should also be tested under changing environmental conditions and for different host plants.

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Supporting Information

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

Fig. S1. Dendrogram depicting the similarity between 29 yeast species isolated from two localities in SE Spain according to growth intensity in media with increasing glucose concentrations.

Table S1. Number of visits and relative abundance of the different flower visiting insect taxa in the censuses in each of the two plant species studied.

Table S2. Factor loadings for the first two PCs based on 29 yeast species growth response (0–4 index) to 82 different tests.

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