

# Species Richness of Yeast Communities in Floral Nectar of Southern Spanish Plants

María I. Pozo · Carlos M. Herrera · Pilar Bazaga

Received: 2 February 2010 / Accepted: 25 April 2010 / Published online: 8 May 2010  
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**Abstract** Floral nectar of insect-pollinated plants often contains dense yeast populations, yet little quantitative information exists on patterns and magnitude of species richness of nectar-dwelling yeasts in natural plant communities. This study evaluates yeast species richness at both the plant community and plant species levels in a montane forest area in southern Spain, and also explores possible correlations between the incidence of different yeast species in nectar and their reported tolerance to high sugar concentrations, and between yeast diversity and pollinator composition. Yeast species occurring in a total of 128 field-collected nectar samples from 24 plant species were identified by sequencing the D1/D2 domain of the large subunit rDNA, and rarefaction-based analyses were used to estimate yeast species richness at the plant community and plant species levels, using nectar drops as elemental sampling units. Individual nectar samples were generally characterized by very low species richness (1.2 yeast species/sample, on average), with the ascomycetous *Metschnikowia reukaufii* and *Metschnikowia gruessii* accounting altogether for 84.7% of the 216 isolates identified. Other yeasts recorded included species in the genera *Aureobasidium*, *Rhodotorula*, *Cryptococcus*, *Sporobolomyces*, and *Lecythophora*. The shapes and slopes of observed richness accumulation curves were quite similar for the nectar drop and plant species approaches, but the two approaches yielded different expected richness estimates. Expected richness was higher for plant species-based than for nectar drop-based analyses, showing that the coverage

of nectar yeast species occurring in the region would be improved by sampling additional host plant species. A significant correlation was found between incidence of yeast species in nectar and their reported ability to grow in a medium containing 50% glucose. Neither diversity nor incidence of yeasts was correlated with pollinator composition across plant species.

## Introduction

The current best guess of planetary diversity at around 14 million species [38] most likely represents a provisional underestimate, given our inability to survey exhaustively all sites and describe taxa before they disappear. Microorganisms contribute an important part to the as-yet-unknown biodiversity because of their vast densities and “misleading” diversity [30, 45]. Molecular tools have dramatically expanded the range of microbial diversity, a significant part of which remained undetected with culture-dependent methods and morphological identification. Despite this, however, exhaustive inventories of microbial communities still remain largely impractical, and under most circumstances, it is necessary to resort to sampling schemes in order to estimate their diversity [22]. Techniques based on species accumulation curves and rarefaction [10, 16] have proven particularly useful to evaluate the relationship between number of species recovered and sampling effort, and could also be used to compare diversities of microbial communities for which samples of different sizes have been gathered.

Microbial diversity differs widely across habitats. In marine microbial communities, for example, published estimates range from hundreds of operational taxonomic units (OTUs) per milliliter in open water [42] to thousands

M. I. Pozo (✉) · C. M. Herrera · P. Bazaga  
Estación Biológica de Doñana,  
Consejo Superior de Investigaciones Científicas (CSIC),  
Avenida Américo Vespucio s/n,  
Isla de La Cartuja 41092 Seville, Spain  
e-mail: maribelpozo@ebd.csic.es

in sediments [21]. Apart from microbial surveys carried out in soil and aquatic environments, other terrestrial environments such as plant-associated microhabitats have been studied less frequently [10, 39]. The diversity of yeast communities, and more specifically those that inhabit floral nectar, still remains a comparatively unexplored subsystem of the microbial communities associated with plants. Although it has been long known that yeasts are frequent and can reach high densities in floral nectar [4, 18, and references therein], relatively little is known on their diversity levels and the factors that influence it [17]. It may be hypothesized that nectar yeast communities are shaped by the interplay between two conflicting forces. On one side, floral nectars, owing to its high energetic content [36], could be considered highly favorable media for microbial growth, but on the other, their high osmotic pressure and frequent presence of secondary compounds [1, 25, 33] could frequently limit the number of yeast species that finally constitute nectar communities [17 and references therein]. In addition to its physical and chemical properties, floral nectar has another particularity. Yeasts need vectors to colonize floral nectar, hence insect visitation history and pollinator identity can be two additional factors influencing the diversity and composition of nectar yeast communities [5, 20, 29]. The main objective of this study was to assess quantitatively the species richness of yeast communities in the floral nectar of a large sample of southern Spanish insect-pollinated plants. This study will also test if observed differences between yeast species in their frequency of occurrence in floral nectar are related to gross physiological differences, by relating the incidence of individual species with their reported tolerance to high osmolarity conditions. In addition, possible correlations across plant species between yeast species richness and pollinator composition will also be explored.

## Materials and Methods

### Study Site and Methods

This study was conducted during May–July 2008 in the Cazorla-Segura-Las Villas Natural Park in Jaén province, southeastern Spain, an area characterized by well-preserved pine-oak montane forests and woodlands (see [18] for additional information). At the time of this study, a large number of plant species were in bloom in the area and thus available for nectar yeast sampling. Additional data, including pollinator censuses and nectar yeast quantification for most plants included in this survey, were also available for the study region. A total of 128 nectar samples collected from flowers of 24 plant species belonging to nine different families were cultured for yeast identification. A complete list of the species

surveyed and their familial affiliations is shown in the “Appendix.” Fabaceae (21% of species), Lamiaceae, Plantaginaceae, Iridaceae (17% of species each), Caprifoliaceae, and Ranunculaceae (8% of species each) were the families contributing most species to our sample. Brassicaceae, Oleaceae, and Solanaceae each contributed a single species to the sample. The species studied here are a subset of those examined microscopically for occurrence of nectar-inhabiting yeasts in the same region by Herrera et al. [18].

Flowering branches, inflorescences, or individual flowers of study species were collected in the field and preserved in a portable cooler inside plastic bags or glass jars for a few hours until taken indoors, and then kept at ambient temperature. Extractions of 1  $\mu$ l nectar samples, using calibrated microcapillaries, were conducted around 12–24 h after field collection. Ten nectar drops per plant species were streaked individually 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 obtained from the resulting colonies following standard morphological criteria described in Yarrow [50]. The D1/D2 domain of the 26S subunit ribosomal DNA was two-way sequenced for all the isolates as described in [27]. Gblocks [7] was used to trim the resulting alignment so that the beginning and ends of the consensus sequences were all the same. Although physiological characteristics are as important as molecular characterization for the identification of yeast species, for practical reasons, we relied exclusively on DNA sequences for identification, which was accomplished by BLAST-querying the GenBank database (last accessed 17 December 2008). Although BLAST searches generally achieved very high similarity scores (usually between 90% and 100%, 91% of the sequences being above 98% similarity score), possible biases in richness estimates caused by the presence of undescribed species were also considered. To this end, this study evaluated OTUs defined on the basis of similarity of DNA sequences [22, 30, 43]. Determination of the number of distinct molecular OTUs occurring in a set of DNA sequences, and assignment of sequences to OTUs, was done with the program DOTUR (Distance-Based OTU and Richness [42]). A PHYLIP (<http://evolution.genetics.washington.edu/phylip.html>)-generated molecular distance matrix was used as input to DOTUR, which assigned sequences to OTUs based on a predetermined distance threshold. Pairs of isolates with molecular distances smaller than the chosen threshold were considered as belonging to the same OTU. A DNA dissimilarity cut-off of 3% was used in the analyses reported below. Although this value is larger than the 1% threshold suggested for species-level rDNA differentiation in yeasts [24; but see 28], this value was chosen because it has been commonly used to distinguish “molecular” fungal

species in environmental studies [37], which would facilitate comparisons with other studies. In any case, the main conclusions of the DOTUR analyses were robust to variations in the OTU discrimination threshold in the range 1–3% (results not shown).

### Statistical Analyses

Densities of up to  $10^5$  yeast cells/ $\mu$ l of nectar are often found in the wild [18], hence direct assessment of true yeast diversity in our samples was impractical, and we had to rely on sampling [22]. Sample-based rarefaction methods, applied to species presence-absence data, were used to assess overall yeast species richness at the plant community level following procedures described by Colwell [9] and Gotelli and Colwell [16]. Nectar drops will provide the elemental “samples” for all analyses, but two different approaches will be adopted to evaluate yeast species richness. In the first one, species occurrence data from all nectar samples will be analyzed together, irrespective of the plant species of origin. This procedure will provide “drop-based” rarefaction curves that assess overall species richness of nectar yeasts at the particular multispecific set of plant species which was sampled. Differences between plant species in life style or floral characteristics could influence their nectar yeast communities, so the second approach considered separately the different host plant species from which nectar samples had been collected. In these analyses, data from all individual nectar samples from the same plant species were combined into a single sample, and “plant species-based” rarefaction curves were obtained. Drop-based and species-based average rarefaction curves were computed with the EstimateS 8.0 program [9], using 50 randomizations and sampling without replacement. Additionally, in order to estimate yeast species richness expected in nectar, the nonparametric estimators ICE and Chao2 were used, because our taxa richness data are based on incidence [8]. Rarefaction generates the expected number of species in a small collection of  $n$  samples drawn at random from the large pool of  $N$  samples [44]. In contrast, richness estimators predict the total richness of a community from samples. This study compared nonparametric estimators with the rarefaction analyses in order to evaluate the accuracy of richness estimates.

To test whether the frequency of occurrence of individual yeast species in floral nectar was related to their osmotolerance, yeasts species were classified into two categories according to their reported growth response in 50% glucose tests, namely, “osmophilic” (positive growth response) and “non-osmophilic” (variable or negative growth response). Physiological data were obtained from Barnett et al. [3] and the on-line CBS yeast database available at <http://www.cbs.knaw.nl/yeast/BioLoMICS.aspx>,

except for *Aureobasidium pullulans* and *Metschnikowia* unknown sp., for which we determined experimentally their responses to variable glucose concentration. Frequencies of occurrence of osmophilic vs. non-osmophilic species in both nectar drops and plant species were compared with the Wilcoxon rank-sum test, using the NPARIWAY procedure in SAS and exact  $P$  value estimation (SAS Institute, Cary, NC, USA).

Correlations across plant species between pollinator composition and yeast incidence were explored using detailed data on pollinator composition obtained in the study region for 12 of the 24 plant species sampled (18, and C. M. Herrera, unpublished data). Only for this analysis, we refer to “diversity” instead of “species richness,” as both species richness and relative species abundances are incorporated into the Shannon–Wiener diversity indices. A principal components analysis was conducted on the variance–covariance matrix of the proportion of flower visits contributed by bumblebees, solitary bees, Lepidoptera, Coleoptera, and Diptera in order to reduce the number of variables describing pollinator composition of each plant species. The first component, which was mainly correlated with the frequency of flower visits by bumblebees and solitary bees (correlations=0.6 and 0.4, respectively), accounted for the 73.2% of total variance. Relationships between nectar yeast incidence and diversity, and pollinator composition, were explored by correlating the frequency of occurrence, yeast abundance, and yeast diversity (Shannon–Wiener index) of yeast cells in nectar, on one side, with the pollinator composition in each plant represented by scores on the first principal components analysis component, on the other. The yeast-related variables mentioned above are also correlated with pollinator diversity calculated by Shannon–Wiener index. For statistical analyses, nonparametric tests were applied (Spearman rank correlation) as implemented in SAS 9.1 statistical package (SAS Institute, Cary, NC, USA) CORR procedure.

## Results

### Observed Species Richness

A total of 128 nectar drops were streaked onto culture media, and the resulting 216 yeast isolates yielded a total of 12 species, comprising both ascomycetous and basidiomycetous yeasts (Table 1). The first group included *Metschnikowia reukaufii*, *Metschnikowia gruessii*, and an unidentified *Metschnikowia*, along with the “black yeasts” *A. pullulans* and *Lecythophora hoffmannii*, while the second group included species of *Rhodotorula*, *Cryptococcus*, and *Sporobolomyces*. Nectar communities sampled had very low species richness, since on average ( $\pm$ SE), only  $1.3 \pm 0.6$  yeast

**Table 1** Plant species from which nectar yeast isolates were achieved, number of DNA isolates, number of DNA isolates containing different yeast taxa

Plant species	DNA sequences, N	<i>Metschnikowia reukaufii</i> , N	<i>Metschnikowia gruessii</i> , N	<i>Aureobasidium pullulans</i> , N	<i>Cryptococcus</i> spp. <sup>a</sup> , N	<i>Rhodotorula</i> spp. <sup>b</sup> , N	<i>Sporobolomyces roseus</i> , N	<i>Lecythophora hoffmannii</i> , N	<i>Metschnikowia</i> sp., N
<i>Anthyllis vulneraria</i>	19	17		2					
<i>Antirrhinum australe</i>	9	5	2	2					
<i>Aquilegia vulgaris</i>	13	13							
<i>Aquilegia cazortlensis</i>	7	7							
<i>Atropa baetica</i>	20	3	14		1	2			
<i>Digitalis obscura</i>	26	2	11	4		7	1		1
<i>Erysimum myriophyllum</i>	1	1							
<i>Erinacea anthyllis</i>	12	8		2	1				
<i>Gladiolus illyricus</i>	7	3	4						
<i>Iris foetidissima</i>	14	14							
<i>Iris pseudacorus</i>	2	2							
<i>Iris xiphium</i>	2				2				
<i>Jasminum fruticans</i>	2		2						
<i>Linaria aeruginea</i>	9	9							
<i>Linaria lilacina</i>	14	14							
<i>Lonicera etrusca</i>	2	2							
<i>Lonicera implexa</i>	11	9	1					1	
<i>Marrubium supinum</i>	10	9	1						
<i>Phlomis lychnitis</i>	6	6							
<i>Prunella grandiflora</i>	7	3	4						
<i>Tetragonolobus maritimus</i>	13	13							
<i>Teucrium pseudochamaepitys</i>	4		4						
<i>Vicia onobrychioides</i>	3	3							
<i>Vicia villosa</i>	3	3							

<sup>a</sup> *Cryptococcus* spp. includes *Cryptococcus aerius*, *Cryptococcus diffluens*, *Cryptococcus uzbekistanensis*, and *Cryptococcus victorinae*

<sup>b</sup> *Rhodotorula* spp. includes *Rhodotorula colostri* and *Rhodotorula mucilaginosa*

species were recovered from each nectar drop. The two dominant species, *M. reukaufii* and *M. gruessii*, were recorded in 73.4% and 29.7% of nectar drops, respectively (Fig. 1). The rest of species were much less frequent, and included *A. pullulans* (found in 7% of nectar samples), *Rhodotorula colostri* (3.9%), *Rhodotorula mucilaginosa* (1.6%), and *Sporobolomyces roseus* (1.6%). Species that were recorded only once (<1% of nectar samples) included *Cryptococcus aerius*, *Cryptococcus diffluens*, *Cryptococcus victoriae*, *Cryptococcus uzbekistanensis*, *L. hoffmannii*, and the unidentified *Metschnikowia* sp. DOTUR analysis of the DNA sequence data for these isolates provided a slightly higher estimate of total species richness, as a total of 18 OTUs were identified at the 3% DNA dissimilarity cut-off. The possibility that additional undescribed species were present in our samples cannot therefore be ruled out.

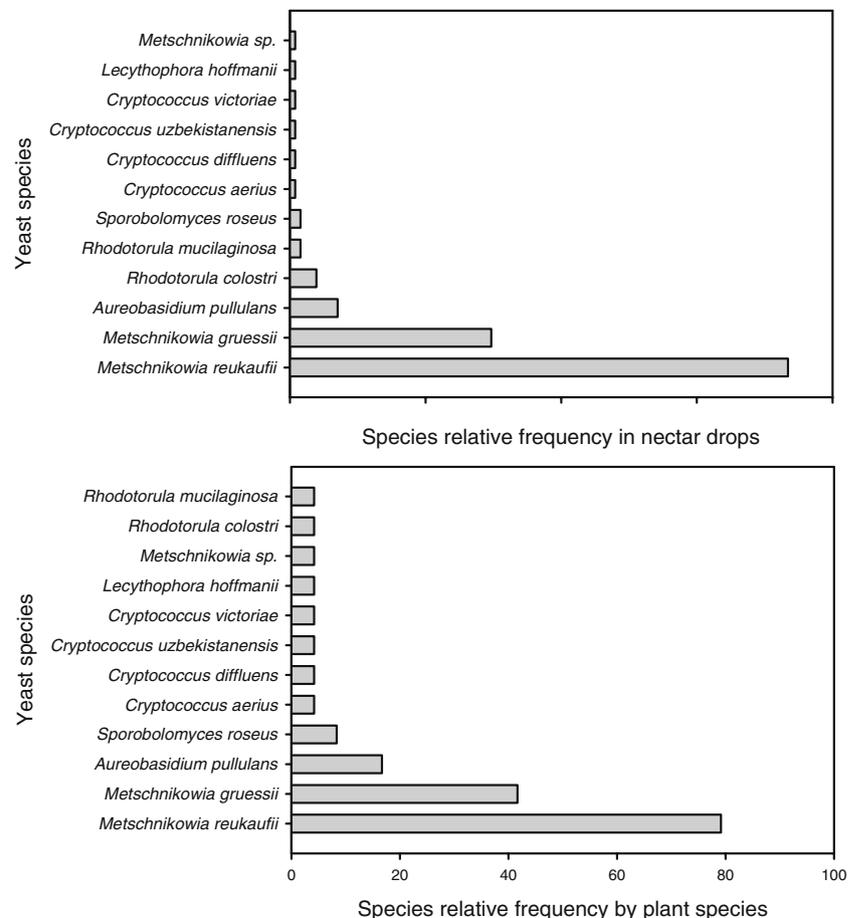
When plant species rather than nectar drops were considered as the samples for analyses, the pattern of yeast community species richness was similar: *M. reukaufii* and *M. gruessii* were isolated from 20 and ten, respectively, of the 24 plant species surveyed. The rest of species occurred in a much reduced subset of plant species. *A. pullulans* and *S. roseus* were found in four and two plant species,

respectively, while the other species occurred in a single plant species each. The most species-rich yeast community occurred in the nectar of *Digitalis obscura* (six species, Table 2), followed by *Erinacea anthyllis* and *Atropa baetica*, with four yeast species each. *Antirrhinum australe* and *Lonicera implexa* harbored three species each, and two species were recovered from the nectar of *Gladiolus illyricus*, *Prunella grandiflora*, *Anthyllis vulneraria*, and *Marrubium supinum*. Only one yeast species occurred in the nectar of each of the remaining species (Table 1).

#### Estimated Species Richness

Although the sampling effort was equivalent for all plant species (ten nectar drops per species), yeast abundance and frequency of occurrence differed widely among species [18], which explains the variation in the number of isolates obtained per plant species (Table 1) and justifies the application of rarefaction-based methods to obtain reliable estimates of total species richness. When the whole sample of nectar drops from all plants species were combined into a single analysis, the species accumulation curve was close to reaching a plateau for the  $N=128$  nectar samples

**Figure 1** Yeast and yeast-like growing species frequency in nectar samples: drop-based (upper panel) and plant species-based (lower panel) approaches



**Table 2** Yeast incidence and species richness, along with pollinator composition for the 12 *Cazorla* species included in the analysis of the relationship between pollination mode and presence of yeasts

Plant species	Yeast frequency (% samples with yeasts)	Yeast density (cells/mm <sup>3</sup> )	Yeast diversity (H')	Pollinator diversity (H')	Pollinator composition (% flower visits)				
					Bumblebees	Solitary bees	Lepidoptera	Coleoptera	Diptera
<i>Anthyllis vulneraria</i>	43.5	1,141	0.1461	0	0.0	100.0	0.0	0.0	0.0
<i>Aquilegia cazortensis</i>	62.9	11,127	0.0000	0.28418	77.0	4.6	18.4	0.0	0.0
<i>Aquilegia vulgaris</i>	60.0	4,356	0.0000	0.14	89.7	10.3	0.0	0.0	0.0
<i>Atropa baetica</i>	17.0	699	0.3971	0.29	59.5	40.5	0.0	0.0	0.0
<i>Digitalis obscura</i>	90.9	3,402	0.6311	0.09	94.0	6.0	0.0	0.0	0.0
<i>Erinacea anthyllis</i>	40.0	2,948	0.4269	0.37	33.5	59.3	7.2	0.0	0.0
<i>Gladiolus illyricus</i>	69.7	5,048	0.2966	0.30	0.0	65.0	33.8	0.0	1.2
<i>Linaria aeruginea</i>	20.0	4,062	0.0000	0.23	0.0	76.9	0.0	23.1	0.0
<i>Lonicera etrusca</i>	13.2	316	0.0000	0.24	16.1	2.6	81.4	0.0	0.0
<i>Marrubium supinum</i>	88.9	584	0.1412	0.31	75.9	16.8	6.6	0.0	0.8
<i>Phlomis lychinitis</i>	45.0	137	0.0000	0.06	96.9	3.1	0.0	0.0	0.0
<i>Vicia villosa</i>	52.6	2,225	0.0000	0.41	46.0	43.9	9.8	0.0	0.3

examined (Fig. 2a). This finding reveals that, although additional rare species are expected to arise by further increasing the total sampling effort on the set of 24 plant species surveyed, results of this survey can be considered as providing a reliable basis for estimating overall yeast species richness in the floral nectar of this set of host species. Species richness estimates obtained from all nectar samples combined were 25.7 (ICE estimator) and 21 species (Chao2 estimator; Fig. 2b), which denotes that our sampling recovered around 50% of the total number of species occurring in the nectar of sampled plant species in the study area.

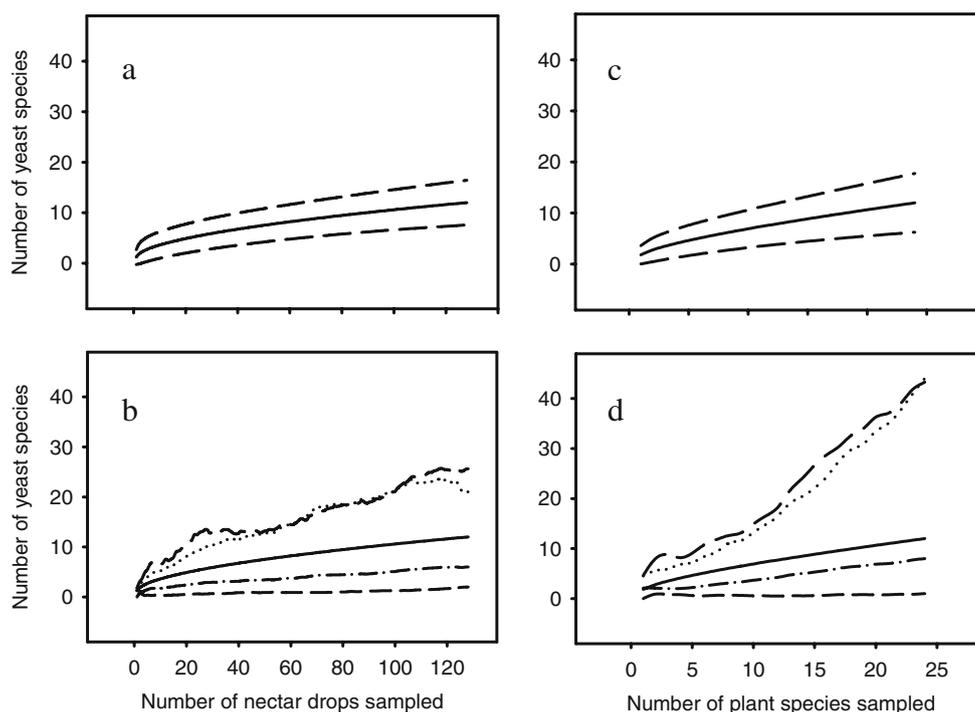
Rarefaction analyses were also conducted using plant species rather than individual nectar samples as the units for the analyses. Using this approach, the yeast species accumulation curve is not linear and does not reach a distinct plateau for the  $N=24$  plant species sampled (Fig. 2c). The two nonparametric richness estimators, ICE and Chao2, gradually drifted apart from the Mao Tau function (observed diversity) with increasing number of plant species (Fig. 2d). The steadily increasing curves shown by these two estimators denote that our sampling of the plant community was able to detect only one third of the total estimated yeast species richness occurring in floral nectar in the regional plant community as a whole.

#### Yeast Community Composition and Osmotolerance

Variation among yeast species in their frequency of occurrence in nectar drops or plant species (Fig. 1) was related to interspecific differences in physiological traits, with osmophilic species occurring most frequently. The magnitude of the effect differed slightly depending on whether species frequencies were computed on the basis of their occurrence in nectar drops or plant species. When frequencies of occurrence were computed in respect to nectar drops, the set of osmophilic yeast species occurred, on average, in 27.7% of samples, while non-osmophilic yeasts occurred in 1.5% of samples, the difference being statistically significant ( $P=0.048$ ; Wilcoxon rank-sum test). Frequency of occurrence across plant species was also significantly greater for the osmophilic yeasts, which on average occurred in 35.5% of plant species, as compared to non-osmophilic ones which occurred in only 4.8% of the plant species surveyed ( $P=0.024$ ).

#### Yeast Diversity and Plant Pollinators

Table 2 summarizes data for the subset of 12 plant species with information simultaneously available on yeast incidence and pollinator diversity and composition. Interspecific variation in yeast diversity and density was not significantly related to quantitative differences in pollinator composition ( $r_s=0.12$ ,  $P=0.71$  in both cases). Pollinator composition, as described



**Figure 2** Species richness vs. species density. **a** Individual-based rarefaction curve (black solid line, Mao Tau function) and 95% confidence intervals (long dash lines) for Cazorla 2008 spring nectar drops dataset. **b** Performance of nonparametric estimators of species richness for Cazorla 2008 spring nectar drops dataset: Singletons (dash-dotted line), Doubletons (medium dash), ICE (long dash), and Chao2 (dotted line), in comparison with rarefaction curve (Mao Tau function, solid line). **c** Individual-based rarefaction curve (black solid

line, Mao Tau function) and 95% confidence intervals (long dash lines) for Cazorla 2008 spring dataset considering plants species surveyed as sampling units. **d** Performance of nonparametric estimators of species richness for Cazorla 2008 dataset by plant species surveyed: Singletons (dash-dotted line), Doubletons (medium dash), ICE (long dash), and Chao2 (dotted line), in comparison with rarefaction curve (Mao Tau function, solid line). Yeast species appearance was based on incidence data

by scores on the first principal component, correlated weakly with yeast frequency, but the relationship did not reach statistical significance ( $r_s = -0.448$ ,  $P = 0.14$ ). No correlations were found between pollinator diversity, as computed with the Shannon index, and yeast variables (frequency, density, and diversity;  $r_s = 0.01$ – $0.18$ ).

## Discussion

As habitat for yeast growth, floral nectar is characterized by high cell densities [12, 18] and low species richness [4, 17], in contrast to other microbial habitats such as aquatic environments that are characterized by high species richnesses [31] and low cell densities [35]. This paper describes a particularly species-poor nectar yeast community, largely composed of only two species (*M. reukaufii* and *M. gruessii*). The rest of species identified were rare in nectar samples, although some of them are common inhabitants in other environments like plant surfaces, soil, air, and water [3, 40]. Species accumulation curves showed that further rare species would appear if more nectar samples from the 24 plant species surveyed were sampled, or if additional

plant species were sampled, but the main conclusion would most likely remain unchanged that nectar yeast communities in our study region are characteristically species-poor.

A number of mechanisms may contribute to the low yeast species richness found in nectar communities [see also 4]. Such factors may include the fact that we have used culture-dependent methods to obtain yeast isolates, irrespective of how the unknown pool of yeast species growing in nectars tolerates the growth medium's conditions. Nevertheless, culture-dependent methods are widely used for the characterization of yeast communities for clinical or ecological purposes, and recent studies on yeast diversity in natural conditions have revealed a better performance of culture-dependent methods over purely molecular techniques like temperature gradient gel electrophoresis or restriction fragment length polymorphism [15, 19]. The existence of undescribed species could be another source of bias in our GenBank-querying species identification method, but results of DOTUR-based analyses tend to rule out this possibility as a major biasing factor. A total of 18 OTUS were identified at the 3% dissimilarity cut-off used, which is not dramatically different from the 12 species identified using BLAST queries. The low yeast species

richness found in this study could also be explained by the fact that we focused exclusively on nectar samples rather than on flowers as a whole. Other surveys of flower-dwelling yeasts yielding longer species lists do not provide detailed information on which floral parts were sampled [27, 29]. In fact, a higher species richness is expected to occur at the level of whole flowers, since flowers harbor contrasting microhabitats for microbial growth such as pollen, perianth surfaces, and nectaries. Soil or plant surfaces could represent macroscales of sampling in relation to microbial body size [14], which might partly account for the high species richnesses revealed by surveys of these microbial habitats.

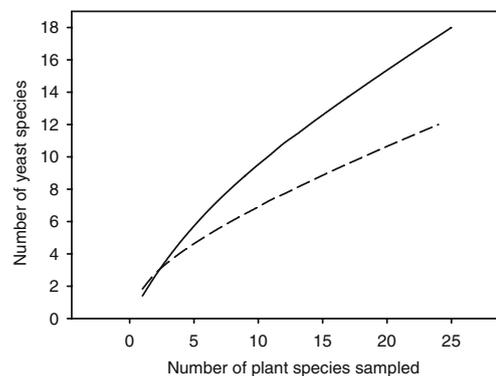
The predominance of ascomycetous over basidiomycetous yeasts found in this study probably reflects that, as a microhabitat, floral nectar favors fermentative, osmotolerant, copiotrophic species. This interpretation is supported by our analyses on the relationship between species occurrence and ability to grow in 50% glucose, and also agrees with some earlier predictions [26]. When interpreting the results of these analyses, however, it must be kept in mind that individual yeast species might be internally heterogeneous in respect to their responses to osmotic stress, and that focusing at the multispecific level as done here misses such source of variation. Additional work on different yeast strains from the different species involved would be needed to explore the potential influence of that effect on our conclusions.

Two groups of yeast species could be distinguished, namely those few present in nearly all nectar samples and the majority that occurred in only a few plant species. The first group includes two specialist species in the *Metschnikowia* clade that have been frequently isolated from flowers, nectars, and pollinators [29]. The second group included generalist species that are found frequently in other microenvironments like leaf surfaces, soil, freshwater, and air. These include species found in decomposing organic matter, phylloplane, and soils (*Aureobasidium*) along with ubiquitous species of *Cryptococcus* and *Rhodotorula*. Additional work is needed to dissect the different ecological origins of yeast species found in floral nectar, discerning which species originate from pollinators' glossae, pollinators body surfaces, corolla inner surface or pollen.

Although rarefaction curves do not provide an estimation of asymptotic species richness [34, 46], they are often used to evaluate sampling adequacy by assessing whether the cumulative number of species reaches a plateau, and to compare observed species richness with figures obtained with nonparametric estimators [10, 11, 47]. Nonparametric richness estimators, infrequently used in microbial diversity estimates [22, and references therein], may be used for inferring true species richness [49]. As shown in this study, observed species richness can be substantially lower than

the values predicted by the ICE and Chao 2 estimators, thus corroborating that observed species accumulation curves will frequently underestimate actual species richness [10]. Nonparametric estimators incorporate information on the distribution of rare species in the dataset (i.e., those represented once, twice, or only a few times), so the fact that the majority of species recovered from our samples were rare account for the marked dissimilarity found here between observed and predicted species richness.

Rarefaction curves did not reach a plateau, thus revealing that additional sampling effort would be needed to assess the true diversity of nectar yeasts in the study area. The total species diversity in a given area can be broken down into within-habitat or alpha-diversity, and between-habitat or beta-diversity [32]. Since alpha-diversity refers to a group of organisms interacting and competing for the same resources or sharing the same environment, results obtained from our drop-based analyses would roughly correspond to this diversity component. In contrast, plant species-based approach would fall conceptually closer to beta-diversity, allowing comparisons between different ecosystems or along environmental gradients [32]. Because of practical limitations, total sampling effort was restricted to 150 nectar drops spread among plant species. If nectar yeast communities were characterized by high beta-diversity, which would imply a high species turnover between plant species, then maximizing the spread of nectar drops across plant species would lead to an optimal sampling design. The use of individual plant species as units of analysis achieved lower accuracy at assessing true diversity at the plant community level than the use of pooled nectar drops, which is not surprising given that the number of samples involved in the nectar drops approach was considerably larger than in the plant species approach. Considering also that each plant species comprises a large array of elemental nectar "habitats," using species as sampling units may provide an excessively coarse scale



**Figure 3** Comparison of sample-based rarefaction curves from this study (dashed line) and a comparable investigation on the nectar-dwelling yeasts from a set of 25 plant species surveyed in Germany by Herzberg [19] (solid line)

for estimating species richness of nectar yeast communities at the habitat level unless a much larger number of nectar samples is collected from each plant species.

Although a number of microbiological surveys of nectar yeasts have been carried out previously [e.g., 4, 13, 19, 23, 41], direct comparisons between our survey and previous work are problematical, since species identifications in the early literature on nectar yeasts was mostly based on traditional microbiological tests [e.g., 6, 41, 48]. The list of genera recorded in our study is closely similar to those reported for *Asclepias syriaca* nectar in North America and a broad survey of nectar yeasts in central European plants [4, 13]. Among recent work using molecular identification methods, the study of Herzberg [19] in Germany remains the most thorough investigation to date on nectar yeasts where the majority of yeast identifications was based on DNA sequences. Results reported in this study and those of Brysch-Herzberg for Germany are similar in that *M. reukaufii* was the commonest species at both sites (65% and 47% of nectar samples in southern Spain and Germany, respectively) followed by *M. gruessii* (23% and 17%). Species of *Cryptococcus* were isolated more frequently in Germany (18% of samples, compared to only 2% in southern Spain). The published data for Germany are amenable to rarefaction analysis, and a comparison of sample-based rarefaction curves for the two regions is shown in Fig. 3. The two species accumulation curves bear strong resemblance and, although similarly low at both regions, observed species richness was slightly higher in Germany than in southern Spain. Although further studies are obviously needed, the provisional conclusion can be drawn from these two studies [see also 6] that European nectar yeast communities are characterized by generally low species richness and a marked numerical dominance by very few species, represented here by *M. reukaufii* and *M. gruessii*. The ecological mechanisms accounting for these patterns remain to be elucidated, but factors related to variable, species-specific colonization opportunities, interspecific antagonistic relationships, and growth limitations imposed by osmotic stress, low nitrogen content, and presence of secondary compounds in the nectar [17] could all play some role in determining the composition and diversity of nectar yeast communities.

**Acknowledgements** We are grateful to Conchita Alonso, André Lachance, and three anonymous reviewers for useful suggestions and discussion, and Pedro A. Tíscar and the Centro de Capacitación y Experimentación Forestal de Vadillo-Castril for essential laboratory space and facilities in Cazorla. R. K. Colwell generously made available the EstimateS software. Permission to work in Sierra de Cazorla was facilitated by the Consejería de Medio Ambiente, Junta de Andalucía. This work was funded 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) to C. M. H. M. I. P. is

supported by a predoctoral grant from the Spanish Ministerio de Educación y Ciencia. M. I. P. is grateful to A. P. López, C. Rosell, and M. Alonso for field help and support and would like to dedicate this article to the memory of Miguel B., for the papers he will never be able to write.

## Appendix

**Table 3** Distribution among families of the 24 angiosperm species whose floral nectar was examined microscopically in this study for the identification of yeasts

Plant family	Species surveyed
Fabaceae	<i>Anthyllis vulneraria</i> , <i>Erinacea anthyllis</i> , <i>Tetragonolobus maritimus</i> , <i>Vicia onobrychioides</i> , <i>Vicia villosa</i>
Lamiaceae	<i>Marrubium supinum</i> , <i>Phlomis lychnitis</i> , <i>Prunella grandiflora</i> , <i>Teucrium pseudochamaepitys</i>
Plantaginaceae	<i>Antirrhinum australe</i> , <i>Digitalis obscura</i> , <i>Linaria aeruginea</i> , <i>Linaria lilacina</i>
Iridaceae	<i>Gladiolus illyricus</i> , <i>Iris foetidissima</i> , <i>Iris</i> <i>pseudacorus</i> , <i>Iris xiphium</i>
Caprifoliaceae	<i>Lonicera etrusca</i> , <i>Lonicera implexa</i>
Ranunculaceae	<i>Aquilegia vulgaris</i> , <i>Aquilegia cazorlensis</i>
Brassicaceae	<i>Erysimum myriophyllum</i>

Familial classification follows [2]

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