

Species coexistence in simple microbial communities: unravelling the phenotypic landscape of co-occurring *Metschnikowia* species in floral nectar

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Summary

Identifying the ecological processes that underlie the distribution and abundance of species in microbial communities is a central issue in microbial ecology and evolution. Classical trade-off based niche theories of resource competition predict that co-occurrence in microbial communities is more likely when the residing species show trait divergence and complementary resource use. We tested the prediction that niche differentiation explained the co-occurrence of two yeast species (*Metschnikowia reukaufii* and *M. gruessii*) in floral nectar. Assessment of the phenotypic landscape showed that both species displayed a significantly different physiological profile. Comparison of utilization profiles in single versus mixed cultures indicated that these two species did not compete for most carbon and nitrogen sources. In mixed cultures, *M. reukaufii* grew better in sucrose solutions and in the presence of the antimicrobial compound digitonin than when grown as pure culture. *M. gruessii*, on the other hand, grew better in mixed cultures in glucose

and fructose solutions. Overall, these results provide clear evidence that *M. reukaufii* and *M. gruessii* frequently co-occur in nectar and that they differ in their phenotypic response to variation in environmental conditions, suggesting that niche differentiation and resource partitioning are important mechanisms contributing to species co-occurrence in nectar yeast communities.

Introduction

Microorganisms constitute the majority of the planet's biological diversity and are crucial for global ecosystem functioning (Finlay, 1997; Pace, 1997). With the advent of novel molecular methods, it has become clear that many microbial ecosystems contain an extremely high phylogenetic diversity and are complex (Torsvik *et al.*, 2002; Venter *et al.*, 2004; DeLong *et al.*, 2006). Identifying the ecological processes that contribute to maintaining microbial diversity has therefore become a central topic in microbial ecology and evolution. However, given the complexity of many natural microbial communities in different terrestrial and aquatic habitats, elucidating the processes that contribute to species abundance and functioning in microbial communities is difficult (Zhou *et al.*, 2002; Pedros-Alió, 2006). The factors that influence the composition and diversity of natural communities of microorganisms could therefore be more easily tracked down in simpler, spatially well-confined communities that are dominated by a relatively low number of species (Rainey *et al.*, 2000).

In the case of floral nectar, microbial communities assemble after microbial species have been transferred to initially sterile nectar samples. Pollinators such as nectarivorous birds or insects are the most likely agents dispersing microorganisms from one flower to the next (Canto *et al.*, 2008; Belisle *et al.*, 2012). Once microbes have entered the flower, the ephemeral nature of nectar samples, on the one hand, favours species with rapid biomass development, whereas the combined effect of low water activity, the presence of plant secondary compounds that may inhibit microbial growth and high C/N ratios that typically characterize floral nectar, on the other hand, may impose severe constraints on microbial

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growth. Tolerance to microaerophilic conditions and ethanol may further affect the ability of nectar-living microbes to survive and to proliferate in floral nectar (Pozo *et al.*, 2012; Lievens *et al.*, 2015). As a result, several potential colonizers may be unable to grow in nectar, resulting in a clear predominance of a limited number of microbial species that frequently occur in the floral nectar of various animal-pollinated plants (Herrera *et al.*, 2010; Pozo *et al.*, 2011).

When two or more species compete for a limited amount of resources, several theoretical models have shown that competitive coexistence requires interspecific trade-offs in species traits (MacArthur, 1972; Tilman, 1982). These trade-offs involve the way in which an organism allocates resources or other factors that limit its fitness. Individuals with similar traits will compete intensely for resources resulting in a fitness disadvantage, whereas individuals that are able to consume different resources will have a competitive advantage and therefore will have higher fitness (Taper and Case, 1992; Schluter, 2000). In this case, it can be hypothesized that microbes that co-occur in floral nectar have evolved different traits that allow stable coexistence, especially because habitat size and available resources are limited within a single nectar sample. However, recent models have shown that species co-occurrence can also lead to phenotypic convergence in the particular case of old coexisting lineages, implying that sometimes convergent selection could preferentially act over selection for trait divergence on those traits mediating interspecific competition (Tobias *et al.*, 2014).

In this study, we tested the hypothesis that phenotypic trait divergence explains co-occurrence of two congeneric yeast species in floral nectar of European plant species. In Western Europe, microbial communities in individual nectar samples are generally species poor (average of 1.3 culturable yeast species), and about 90% of the culturable yeast isolates obtained from floral nectar belong to the ascomycetous yeasts *Metschnikowia reukaufii* and *M. gruessii* (Giménez-Jurado, 1992; Brysch-Herzberg, 2004; Pozo *et al.*, 2011; Alvarez-Perez and Herrera, 2013). Preliminary evidence indicates that these two species frequently co-occur in floral nectar, as they often have been found simultaneously in nectar of the same host species and individual plants, and often even in the same floral nectar sample (Pozo *et al.*, 2011). To elucidate, in more detail, the mechanisms that lead to the co-occurrence of these microbes, we first provide quantitative evidence about the sympatric occurrence of the two *Metschnikowia* species at the regional scale by examining the frequency of co-occurrence in the floral nectar of a wide range of bumblebee-pollinated European plant species. Second, we used a series of phenotypic tests to investigate whether isolates of the two species sampled in

a common set of host plant species in the same study area displayed different phenotypic profiles. Third, detailed experiments were conducted that provided insights into the establishment process of co-occurring yeasts immediately after they have been dispersed into a flower. More specifically, we assessed whether species co-occurrence affected the performance of *M. reukaufii* and *M. gruessii* strains using test conditions that mimic the colonization process of microorganisms in nectarines across the ephemeral life span of a flower, i.e. starting from the very early stages of the colonization process until wilting of the flower. Taken together, our results provide novel insights into the nature of the interaction between this species pair in naturally occurring nectar communities, and more generally on the mechanistic basis of microbial co-occurrence in patchy, ephemeral natural habitats.

Results

Regional-scale co-occurrence of Metschnikowia species in floral nectar

Metschnikowia species were detected in 868 samples out of a total of 1656 (52.4%) individual nectar samples analysed microscopically. The two yeast species co-occurred in 212 samples, and the remaining 656 harboured either *M. gruessii* (370) or *M. reukaufii* (286) alone. The two yeast species were found in 28 out of the 63 plant species analysed. In three plant species (*Silene colorata*, *Teucrium polium* and *Teucrium rotundifolium*) both species were isolated, although they did not occur simultaneously in the same nectar samples. The remaining 32 plant species harboured *M. reukaufii* (16) or *M. gruessii* (16) exclusively (Fig. 1). The co-occurrence of the two yeast species was not clumped in our phylogeny (Fig. 1), not even when we considered co-occurrence of the two yeast species in the same or different nectar sample of the same plant species [$D \approx 1$, probability of $E(D)$ resulting from Brownian phylogenetic structure ≈ 0]. Using the continuous trait 'relative number of nectar samples with the two yeasts species', no phylogenetic signal was obtained ($K = 0.14$, $P = 0.21$).

The frequency of co-occurrence in floral nectar samples varied significantly across plant families ($F_{18,1637} = 5.91$, $P < 0.0001$) and plant species ($F_{28,1593} = 4.62$, $P < 0.0001$). Families with a higher probability of harbouring both species in individual nectar samples were the Asteraceae, Boraginaceae, Caprifoliaceae, Iridaceae, Lamiaceae, Plantaginaceae and Solanaceae. In contrast, *M. reukaufii* dominated the nectar communities in the families Amaryllidaceae, Caryophyllaceae, Ericaceae and Ranunculaceae, whereas *M. gruessii* was predominant in nectar samples belonging to the Asteraceae and the Lamiaceae (Fig. 1).

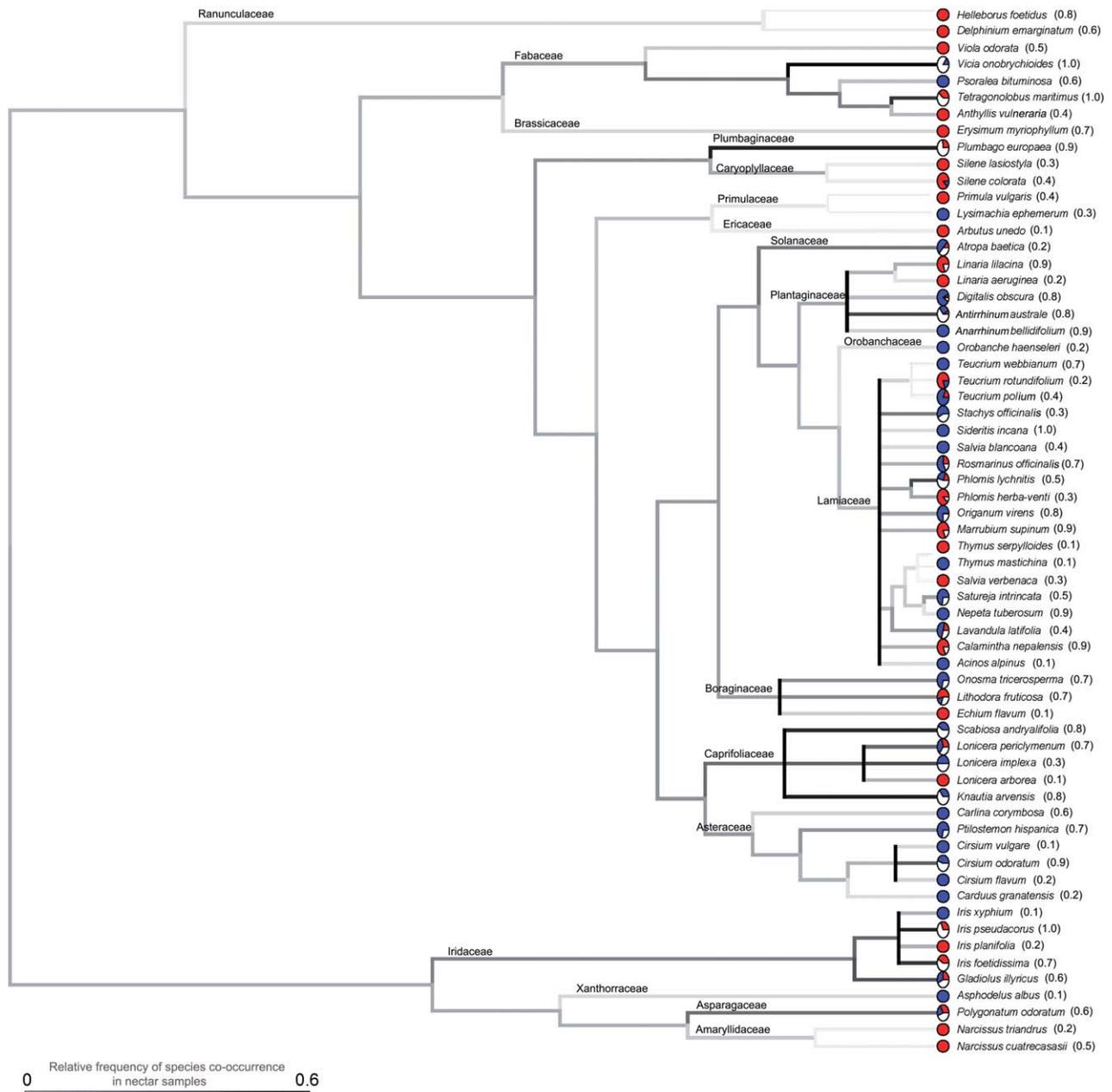


Fig. 1. Phylogenetic relationships of plant species whose nectar was analysed microscopically to check species co-occurrence. Pie charts depict the relative abundance of individual samples with either *M. gruessii* (blue), *M. reukaufii* (red) or both species (white) with respect to the total number of samples with yeasts analysed per plant species. Different colours in branches were used to denote increments (see figure legend) in the relative frequency of nectar samples in which the two species coexisted at the higher taxonomical levels, using the function plotBranchbytrait as implemented in R package 'phytools'. Of a total of 1656 individual nectar samples that were analysed microscopically, 868 (52.4%) harboured yeasts. Numbers in parentheses after species names denote the relative frequency of nectar samples containing yeasts per plant species.

Phenotypic differences between the two *Metschnikowia* species

The overall phenotypic profile of the isolates tested differed significantly between the two yeast species [Pseudo- F = 82.61, degrees of freedom (df) = 1,

P = 0.001, Fig. 2]. Non-metric multidimensional scaling (NMDS) analysis for every compound family further showed substantial differences in the phenotypic profile of the two yeast species. We found significant effects for yeast species for carbon utilization (Pseudo- F = 113.93, P = 0.001), nitrogen utilization (Pseudo- F = 16.97,

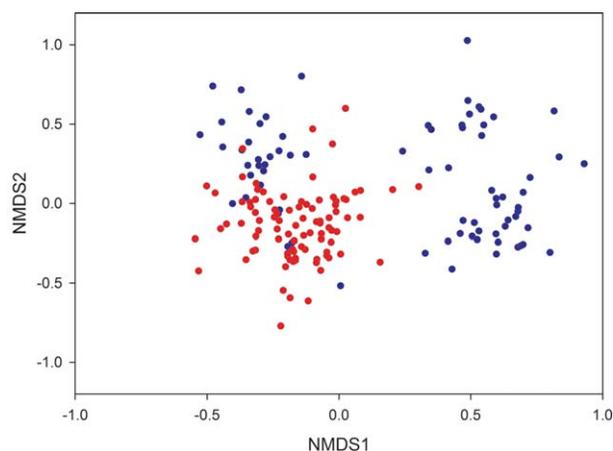


Fig. 2. NMDS ordination of the growth of the two studied yeast species *M. gruessii* (blue) and *M. reukaufii* (red) in 47 phenotypic tests (see Table S2 for list of conditions tested).

$P = 0.022$), inhibitor resistance (Pseudo- $F = 83.76$, $P = 0.001$) and the hydrolysis tests (Pseudo- $F = 50.13$, $P = 0.001$). Yeast strain response on osmotic stress had no significant effect for yeast species (Pseudo- $F = 1.38$, $P = 0.16$).

Non-parametric univariate analyses showed significant differences between *M. reukaufii* and *M. gruessii* for 31 out of the 47 phenotypic tests (Table 1). In agreement with the multivariate analyses, tests related to sugar and nitrogen assimilation, hydrolysis of lipids and proteins and growth inhibitors showed significant differences between the two species, whereas almost all tests related to low water activity showed no significant difference in growth response between both species. In general, growth of *M. reukaufii* was more intense than that of *M. gruessii* (i.e. for all tests for which significant differences were found). For the few tests in which *M. gruessii* grew better than *M. reukaufii*, differences were not statistically significant (Table 1). In particular, *M. reukaufii* significantly outperformed *M. gruessii* on 23 of 27 carbon sources (the exceptions were raffinose, melezitose, sorbose and xylose), two out of the three tested nitrogen sources (lysine, cadaverine) and tolerance to inhibitors (digitonin, CTAB 10 ppm, HCl and fluorocytosine). Although both species showed vitamin dependence, *M. reukaufii* strains showed a higher background growth on vitamin-free medium. Additionally, whereas no *M. gruessii* strain was able to hydrolyse casein, almost 23% of all tested *M. reukaufii* strains were able to do so. Following an incubation period of 12 days, 2.6% of *M. gruessii* strains grew in 50% glucose and in 50% fructose, compared with none for the *M. reukaufii* strains. No significant overall difference in intraspecific phenotypic variability was observed ($\chi^2 = 0.11$, $P = 0.74$). However, differences in variability were observed for some specific tests (Fig. S1). In par-

ticular, phenotypic variability for nitrogen was significantly higher for *M. reukaufii* strains than for *M. gruessii* strains ($\chi^2 = 3.86$, $P = 0.05$).

Co-occurrence of Metschnikowia species in floral nectar at the local scale

In co-cultures, both species showed significantly higher growth than in pure cultures for 16 out of the 45 parameters tested (Table S4, Fig. 3). Those parameters comprised tolerance to inhibitors, growth on vitamin-free medium, and glucose 50%, along with several carbon sources listed in Fig. 3. In contrast, the growth of both species was lower in mixed cultures than in pure cultures and in alkaline pH, lipids and CTAB 10 (Table S4).

More detailed experiments using dual cultures in nectar-mimicking conditions indicated that clonal lineages of *M. reukaufii* and *M. gruessii* can co-occur as both species were still alive and multiplying after 2 days. Growth of each species in co-cultures versus single cultures did not differ at high and low pH (Fig. 4). The same effect was found for growth on glucose in the particular case of *M. reukaufii* (Fig. 4). However, *M. gruessii* grew significantly worse in co-culture conditions on sucrose and in the presence of the plant secondary compound digitonin ($T = -6.39$, $df = 126$, $P < 0.0001$; and $T = -6.87$, $df = 126$, $P < 0.0001$; respectively). In contrast, *M. gruessii* grew better in co-culture conditions on fructose and glucose ($T = 2.01$, $df = 126$, $P = 0.046$; and $T = 2.23$, $df = 126$, $P = 0.02$; respectively) and we also found a higher growth for co-cultures than for single cultures of *M. reukaufii* for sucrose and digitonin media ($T = 5.68$, $df = 126$, $P < 0.0001$; and $T = 5.42$, $df = 126$, $P < 0.0001$; respectively). Furthermore, judging from the counts of the single species growing in co-culture conditions we found that *M. reukaufii* consistently grew more than *M. gruessii* ($T = -4.94$, -6.82 , -2.18 , -7.80 , -5.26 , -3.53 ; $df = 126$, $P < 0.0308$; for sucrose, glucose, fructose, digitonin, low and high pH respectively). The same trend was obtained in single cultures (Fig. 4), except for sucrose media, in which *M. gruessii* grew better (Fig. 4, $T = 3.25$, $df = 126$, $P = 0.0015$) and digitonin, where the single cultures of the two species reached an equivalent growth (results not shown).

Discussion

Co-occurrence of closely related yeast species

Floral nectar is an aqueous solution largely dominated by a few sugars, such as sucrose, glucose and fructose (Nicolson and Thornburg, 2007; Pozo *et al.*, 2014). Because of the rapid proliferation of nectar yeasts, the relative amount of total sugars sharply decreases

Table 1. Differences in growth response (except casein hydrolysis, see Experimental procedures) between yeast species (*M. reukaufii* and *M. gruessii*)

Category	Test	χ^2	<i>P</i>	% Δ Mr – Mg
Carbon sources	Raffinose	0.30	0.5824	–10.06
Carbon sources	Galactose	63.09	< 0.0001	58.72
Carbon sources	Trehalose	45.05	< 0.0001	57.13
Carbon sources	Maltose	18.46	< 0.0001	47.92
Carbon sources	Melezitose	4.86	0.0275	12.37
Carbon sources	α -Methyl glucoside	14.56	0.0001	29.22
Carbon sources	Cellobiose	33.75	< 0.0001	41.20
Carbon sources	Salicin	55.48	< 0.0001	54.76
Carbon sources	Sorbose	2.27	0.1300	28.65
Carbon sources	Rhamnose	12.15	0.0005	24.19
Carbon sources	Xylose	0.04	0.8350	–31.60
Carbon sources	Ribose	61.25	< 0.0001	86.22
Carbon sources	Glycerol	21.10	< 0.0001	28.63
Carbon sources	Ribitol	26.59	< 0.0001	17.25
Carbon sources	Xylitol	54.81	< 0.0001	57.61
Carbon sources	Mannitol	69.35	< 0.0001	63.22
Carbon sources	Glucitol	53.59	< 0.0001	45.52
Carbon sources	Succinic acid	69.14	< 0.0001	63.54
Carbon sources	Citric acid	58.05	< 0.0001	70.94
Carbon sources	Malic acid	25.78	< 0.0001	39.22
Carbon sources	Gluconic acid	35.50	< 0.0001	36.53
Carbon sources	Gluconolactone	64.77	< 0.0001	59.36
Carbon sources	Glucosamine	92.91	< 0.0001	65.51
Carbon sources	N-acetyl glucosamine	40.82	< 0.0001	53.85
Carbon sources	Arbutin	80.19	< 0.0001	68.57
Carbon sources	4-Nitrophenyl- β -D-glucopyranoside	61.87	< 0.0001	71.78
Carbon sources	Rebaudioside	61.43	< 0.0001	59.65
Various	Casein hydrolysis	49.51	< 0.0001	100.00
Various	Vitamin free	18.85	< 0.0001	30.77
Various	Tween 80 hydrolysis	5.25	0.0021	–23.86
Various	Alkaline pH	7.41	0.0065	–15.92
Inhibitors	Digitonin	20.67	< 0.0001	47.36
Inhibitors	CTAB10	16.57	< 0.0001	22.55
Inhibitors	CTAB50	1.73	0.1886	94.34
Inhibitors	Deoxycholate	10.51	0.0012	–86.46
Inhibitors	Sodium carbonate	2.80	0.0942	62.39
Inhibitors	HCl	81.25	< 0.0001	69.24
Inhibitors	5-Fluorocytosine	66.91	< 0.0001	57.01
Inhibitors	Ethanol	1.74	0.1800	–22.46
Nitrogen	Ethylamine	5.62	0.0177	86.09
Nitrogen	Lysine	59.10	< 0.0001	92.44
Nitrogen	Cadaverine	22.84	< 0.0001	74.22
Low water activity	NaCl 5%	4.33	0.0375	87.09
Low water activity	NaCl 10%	0.00	1.0000	0.00
Low water activity	50% sucrose	2.29	0.0696	–9.47
Low water activity	50% fructose	3.94	0.0473	–100.00
Low water activity	50% glucose	1.26	0.2609	–100.00

Statistical significance is shown in bold fonts after Bonferroni correction. Last column denotes the relative performance shown by *M. gruessii* in comparison with *M. reukaufii*: positive percentages denote superior performance by *M. reukaufii*, and vice versa.

in exposed flowers (Herrera *et al.*, 2008). Species co-occurrence in resource-limited habitats such as floral nectar is therefore more likely to occur when co-occurring species differ in their resource utilization. In this paper, we tested this hypothesis for the yeast species *M. reukaufii* and *M. gruessii*, which often co-occur in floral nectar in Europe. Because the two species occupy the same microhabitat and owing to an unclear understanding of their phenotypic differences, *M. reukaufii* and *M. gruessii* were once regarded as members of a single species (see Brysch-Herzberg, 2004 and references therein).

However, taxonomic investigations based on DNA have led to the distinction between the two species (Giménez-Jurado, 1992), providing a unique opportunity to investigate in detail the mechanisms contributing to co-occurrence of two closely related species in an ephemeral habitat such as floral nectar.

Our detailed observations have confirmed that the two species are morphologically distinguishable, and that at a regional scale they frequently occur simultaneously in floral nectar, especially in certain plant families. However, our analysis of phylogenetic signal strength revealed that

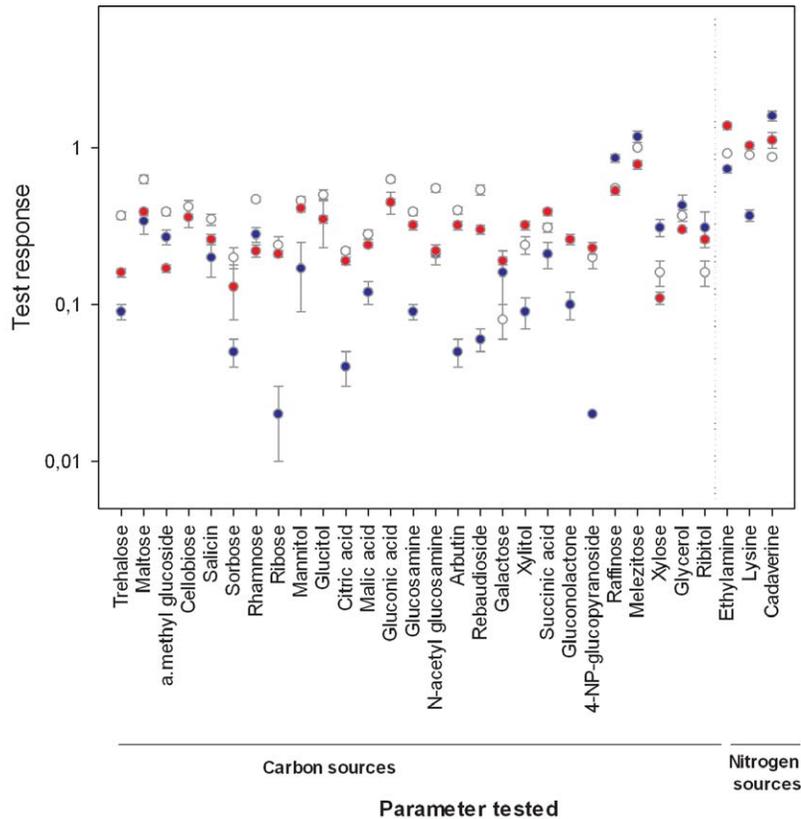


Fig. 3. Microbial growth in carbon and nitrogen media measured in *M. gruessii* (blue circles) and *M. reukaufii* (red circles), and their mixed cultures (white circles) (displayed in logarithmic scale). Each point is the mean value obtained for a species at a given temperature (24°C), involving multiple strains and experiments, and vertical bars represent standard errors. Tests along the x-axis are ordered depending on response type: higher in co-culture, *M. reukaufii* and *M. gruessii* respectively.

the co-occurrence of the two *Metschnikowia* species was not clumped. This is likely due to the fact that the different plant families were not equally represented in our dataset. Sympatric distributions have been observed for other floricolous yeasts. For example, the asexually reproducing yeast *Candida ipomoeae* is frequently found in sympatry with closely related species of the large-spored *Metschnikowia* clade in the Neotropics, where they occupy microhabitats associated with beetles and the flowers visited by them (Wardlaw *et al.*, 2009). However, similar to *M. reukaufii* and *M. gruessii*, one species (in this case *C. ipomoeae*) shows a much greater geographic range than the other species, which raises the intriguing question of the specific mechanisms that determine why both species can occur together, but at the same time allow them to show important differences in distribution area.

Sources of intraspecific variation

The *Metschnikowiaceae* clade is thought to have originated in the Late Cretaceous (71.7 Ma) and its evolution appears to have been driven by complex and dynamic habitat transitions, with repeated and independent origins of angiosperm-associated habitats (Guzmán *et al.*, 2013). Most species are terrestrial and form diverse mutualistic

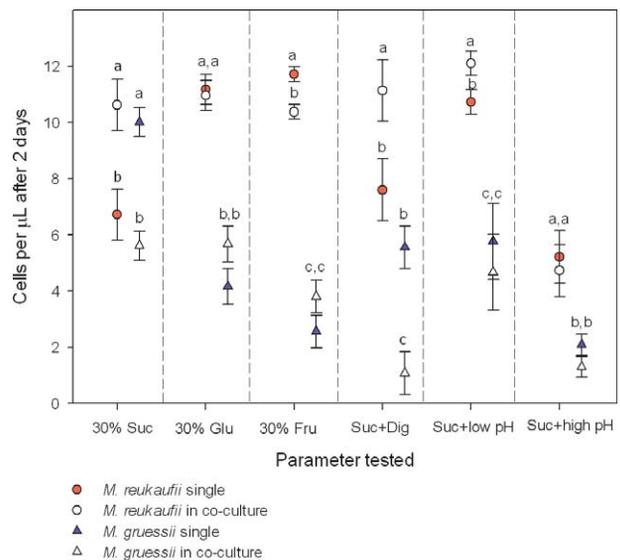


Fig. 4. Final growth after 2 days, measured as cells per μL , in nectar-mimicking parameters measured in *M. gruessii* and *M. reukaufii* in single culture versus co-culture of the two species. Each point is the mean value obtained for a species, involving two strains per species and two replicas per strain, and vertical bars represent standard errors. The letters denote comparisons within each species across different conditions (mixed or single) and comparisons within single and mixed conditions across species. Means with the same letter were not significantly different at $P < 0.05$.

symbioses with a preponderance of associations with angiosperms and their associated insects (Lachance, 2011a,b). Interactions with angiosperms are pointed out as important factors driving macro-evolutionary processes in these lineages. *M. reukaufii* and *M. gruessii* populations show a high genetic diversity, in spite of their clonal mode of reproduction in nature (Herrera *et al.*, 2011; 2014). This genetic diversity is favoured by the contrasting habitats that these two species occupy, namely the tongue of bumblebees and the phylloplane or nectar of different host plants (Herrera *et al.*, 2011; 2014). As a result, the two species were expected to have broad ecological niches with a high degree of phenotypic plasticity (Roughgarden, 1972; Sultan and Spencer, 2002; Baythavong, 2011). Our analyses of the phenotypic profiles support this hypothesis and indicate that the nectar-living *M. gruessii* and *M. reukaufii* can explore a wide phenotypic landscape. The visual analysis of the phenotypic landscape explored by the two species shows a higher dispersion in *M. gruessii*. This species also showed systematically higher percentages of intraspecific variance for several carbon sources. We could hypothesize that differences in nectar physicochemical factors that are relevant to the phylogeny of the plant (Nicolson and Thornburg, 2007) could account for the within-species variation observed in these two yeast species. This finding also raises some methodological warnings. Studies aiming to elucidate the phenotypic and genotypic diversity of nectar-living *Metschnikowia* should carefully consider the origin and the number of strains per individual species they include in the experimental design.

Resource partitioning

A large set of phenotypic tests using various substrates was conducted to investigate whether resource partitioning can be invoked as a possible mechanism contributing to the frequent co-occurrence of the nectarivorous yeasts *M. reukaufii* and *M. gruessii* once they have colonized floral nectar. Using both univariate and multivariate analyses, our results clearly showed that the two species occupied virtually non-overlapping regions in the reduced dimensionality of the phenotypic space obtained, indicating the two species could be readily separated phenotypically under the experimental conditions that the species were exposed to. Interspecific partitioning of nutritional resources has long been proposed as a mechanism that promotes species co-occurrence in macroorganisms (Schoener, 1974). More recently, however, there has been compelling evidence that microbial communities, in contrast to the 'everything is everywhere' principle, could be shaped by temporal segregation, which may limit competition for limited available resources (Carreiro and Koske, 1992; Sweeney *et al.*, 2004). However, most of those

studies, which have exclusively focused on thermal tolerance limits, have obtained limited success at delimiting species phenotypically (Sweeney *et al.*, 2004). Although *M. reukaufii* and *M. gruessii* may show slight differences in their thermal tolerance limits (Pozo *et al.*, 2012), the species coexist during a large part of the year, such that temporal segregation does not seem to play an important role in these communities.

Because of the specific goal of this study, our screening scored the growth of the two species at the short term, thus mimicking the average floral life span in spring–summer blooming species in a Mediterranean climate (Pozo and Herrera, results not shown). As a result, both species may fail to show a positive response in some of the standard compounds used to assess yeast taxonomy (Lachance, 2011b). We have found that *M. reukaufii* performed better in several parameters than *M. gruessii*. It is therefore surprising that *M. gruessii* is still so prevalent in Europe, given that it is mostly outcompeted by *M. reukaufii* under the majority of conditions tested. Nectar yeast species co-occurrence might be due to habitat selection, in which members of a species prefer to remain in that part of the environment to which it is best adapted (Ackerly, 2003). By means of this kind of stabilizing selection, *M. gruessii* might be prevented to leave their ancestral niche in spite of co-occurring with more competitive and widespread sympatric species, such as *M. reukaufii*. In addition, temporal differences in colonization might explain the frequent occurrence of *M. gruessii* in nectar if *M. reukaufii* first creates nectar conditions that are suitable for later colonizers (Peay *et al.*, 2012; Vannette and Fukami, 2014). However, *M. gruessii* tended to show a greater performance in nectar-like media such as 50% or 30% sucrose (Pozo *et al.*, 2014), and the two species did not differ significantly in their high sugar concentration tolerance limits (Pozo *et al.*, 2012; Lievens *et al.*, 2015).

The possibility could not be ruled out, however, that mixed cultures found in the field could be due to frequent re-inoculation of the less competitive species, such as *M. gruessii*. The typical airplane cell morphology of *M. gruessii* has been interpreted as a successful adaptation that promotes dispersal through bumblebee hairy tongues (Brysch-Herzberg, 2004). Frequent re-inoculation, as a result of a better dispersal strategy, might also explain the higher frequency of *M. gruessii* in late spring and summer, when flowers are visited more frequently (Pozo *et al.*, 2012).

Local-scale coexistence: competition versus facilitation

For the majority of parameters tested, we did not find any evidence that one species outcompeted the other. This does not mean that they do not compete actively for

resources, but at least, in a period that encompasses the lifespan of a single flower (like our trials, where strains performed normally in a 2 day response), when resources are still available, the two species still attained a positive growth response in co-cultures as compared with the initial cultures. However, judging by the species co-occurrence assays, we found a facilitation rather than a competition effect under some conditions, meaning that the strains benefitted each other. Our nectar-mimicking assays in liquid medium showed that both species were able to grow simultaneously, although *M. reukaufii* always reached a higher abundance. Instead of competitive dominance of a single species, we found that *M. guessii* facilitates *M. reukaufii* growth in the sucrose test medium as well as in the presence of digitonin in detriment of its own growth. On the other hand, *M. reukaufii* was found to facilitate the growth of *M. guessii* in the hexose test solutions. Synergistic interactions were also found for *M. pulcherrima* strains found on the skin of grapes (Csutak *et al.*, 2013), and yeast communities described at the cactus phyllosphere (Lachance and Starmer, 1998). Facilitation effects found for sugars and inhibitors such as digitonin might result from species-specific physical changes, depending on enzymatic complementation and the secretion of extracellular factors. For instance, it is possible that interactions between secreted components might result in increased viscosity in the matrix, by which the diffusion of the inhibitor and/or its activity would be reduced (Burmolle *et al.*, 2006). Also, protection of species by the most resistant species could be proposed (Leriche *et al.*, 2003).

We also tested the idea that growth of one species would negatively affect the growth of the other species. Production of killer toxins by some yeast strains has been shown to destroy the cells of other yeast strains, usually of other species (Golubev, 1998; Lachance and Starmer, 1998). Such killer toxins have been described in *M. pulcherrima* (Farris *et al.*, 1991), although it is not clear whether the inhibitory action may not have been caused by pulcherrimin, an iron-scavenging pigment (Sipiczki, 2006). *M. reukaufii* and *M. guessii* are not known to produce killer toxins or pulcherrimin, and we have not observed competition by interference in pairs of strains.

Model-adjusted cell densities after 2 days of incubation varied among the nectar-mimicking parameters that we tested. However, they were consistently lower than the average of 100 cells μL^{-1} of nectar sample that we have found by analysing yeast cell density in 63 plant species in Cazorla region. This difference may be explained by the higher complexity of natural nectar samples, as floral nectar is not only composed by sugars but also by other minor elements, such as organic acids, volatile oils, vitamins, proteins, amino acids and plant secondary compounds that may increase growth. Moreover, open flowers

can be re-inoculated by subsequent pollinator visits, further increasing yeast cell density.

Conclusions

Mechanisms involved in the assembly and functioning of ecological communities of plants or animals have been extensively investigated in the past. On the contrary, less is known about the processes that shape microbial communities, most probably due to the great complexity of the majority of these microbial communities in natural ecosystems. In this paper, the establishment of a simple microbial community, i.e. consisting of a few nectar yeasts, was used as a model to test the mechanisms behind the possible coexistence of two nectar yeast species. Overall, our results provided clear evidence that *M. reukaufii* and *M. guessii* frequently co-occur in nectar and that they differ in their phenotypic response to variation in environmental conditions, suggesting that niche differentiation and resource partitioning are important mechanisms contributing to species co-occurrence in nectar yeast communities. Additional work is needed to elucidate if this mechanism also leads to the stable co-existence of this species pair in more complex experimental conditions than the ones tested here. In particular, a follow-up study on changes in nectar traits caused by both yeast species simultaneously would be of interest, as this may impact other mutualistic partners, such as plant pollinators.

Experimental procedures

Study species

Metschnikowia reukaufii and *M. guessii* are ascomycetous yeasts associated with floral surfaces, floral nectar and flower-visiting insects such as bumble bees (Brysch-Herzberg, 2004). The yeasts are brought to flowers by foraging insects, most often solitary and social bees (Brysch-Herzberg, 2004; Herrera *et al.*, 2010; Pozo *et al.*, 2012). Vegetative cells found in flowers are diploid and proliferate profusely by multilateral budding, reaching densities in floral nectar $> 5 \cdot 10^4$ cells μL^{-1} within 2–4 days of colonization. Under stringent culture conditions, they may reproduce sexually with the production of asci and ascospores (Giménez-Jurado *et al.*, 1995; Miller and Phaff, 1998), but sexual reproduction does not seem to occur naturally in the flower-living populations of our southern Spanish study area (see below) (Pozo and Herrera, pers. obs.).

Microscopic survey

In order to assess the sympatric occurrence of both yeast species in floral nectar, a total of 1656 nectar samples (harvested from 2008 to 2010) from 63 plant species growing in the Sierra de Cazorla-Segura-Las Villas natural Park, a well-preserved montane forest in SE Spain, were examined microscopically for the presence of both species. An average

(±standard error) of 34.1 ± 2.4 nectar samples were examined per plant species (range = 10–150 samples/species). Each nectar sample originated from a different flower that had been previously exposed to natural pollinator visitation, and was obtained following the methods described in detail by Herrera and colleagues (2009). *M. reukauffii* and *M. gruessii* show characteristically different cell morphologies when growing in sugar-rich solutions such as floral nectar (Giménez-Jurado, 1992; Kurtzman *et al.*, 2011). More specifically, *M. reukauffii* cells fully colour blue by adding lactophenol cotton blue while *M. gruessii* does not. The latter also shows characteristic cruciform cell configurations (Brysch-Herzberg, 2004). Altogether, these specific features enabled us to determine whether both yeast species, or one or the other were present in a single nectar sample by microscopical observation. Further, identifications were corroborated by molecular methods for a substantial fraction of nectar samples following the methods described below.

Strain isolation, identification and storage

In addition to the microscopic survey outlined earlier, yeast strains were isolated from several nectar samples collected over different years (2008, 2009 and 2013) from 24 plant species growing in the Sierra de Cazorla-Segura-Las Villas Natural Park. Yeasts were isolated using the methods described by Pozo and colleagues (2011). Briefly, isolation consisted of streaking nectar onto yeast extract glucose chloramphenicol (YGC hereafter, Sigma Aldrich) agar plates followed by incubation, purification and identification of the obtained isolates (one isolate per colony morphotype). In total, this resulted in 681 *Metschnikowia* isolates, from which 93 *M. reukauffii* and 78 *M. gruessii* isolates were selected for further study. These 171 isolates originated from nectar of 10 plant species in which both yeast species have been isolated, namely *Antirrhinum australe*, *Aquilegia vulgaris*, *Atropa baetica*, *Digitalis obscura*, *Gladiolus illyricus*, *Marrubium supinum*, *Phlomis lychnitis*, *Prunella grandiflora*, *Teucrium pseudochamaeipyitis* and *Vicia onobrychioides* (Table S1, Supporting Information). All isolates originated, with a few exceptions, from flowers of different plant individuals.

Isolates were identified on the basis of both morphological characteristics and by two-way sequencing of the variable D1/D2 domain of the large subunit (26S) ribosomal RNA gene using primers NL1 and NL4 (Pozo *et al.*, 2011). Consensus sequences were compared with the type strain sequences (CBS 7657 for *M. gruessii* and CBS 5834 for *M. reukauffii*) obtained from GenBank (accession numbers U45737 and U44825 respectively). Isolates were stored in Microbank™ preservation system vials at –80°C and then transferred to 96-well plates with 40% glycerol in sterile deionized water.

Selection of phenotypic profiling parameters and preparation of media

The test parameters and test concentrations used for the phenotypic profiling were selected based on unpublished data obtained in a pilot study on 100 *Metschnikowia* strains. Test parameters included in the pilot study consisted of 44 carbon sources, five nitrogen sources, 20 inhibitors and a

number of factor requirement tests. Additionally, strains were tested for osmotolerance, halotolerance, hydrolysis capacity, acid production and colour reaction (diazonium blue B reaction and starch production). These parameters represent standard tests commonly used in yeast systematics (Kurtzman *et al.*, 2011). Based on the results, 47 tests that were considered relevant for this study were selected, representing parameters related to assimilation of carbon (27 parameters) and nitrogen (three), low water activity (three osmotolerance and two halotolerance tests), resistance to inhibitors (eight), growth at high pH (nine), growth on a vitamin-free medium and hydrolysis of casein or Tween 80 (Table S2).

For inhibitor and halotolerance tests, YM agar (0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1% glucose, 2% agar) was used as basic medium, supplemented with the test compound (for tested concentrations, see Table S2). Osmotolerance was evaluated using 50% w/w glucose, 50% w/w fructose and 50% w/w sucrose, representing the three main sugars in floral nectar (reviewed in Nicolson and Thornburg, 2007) supplemented with 1% yeast extract. For halotolerance, we used two concentrations of NaCl (5% and 10% w/w). For carbon assimilation, we tested 1% of each test compound in yeast nitrogen base (YNB), with YNB–glucose 1% and YNB as the positive and negative reference plates respectively. For nitrogen sources, we used 0.06% w/w in yeast carbon base (YCB); YCB medium with no additional nitrogen source was used as reference.

All ingredients used in the preparation of the media were purchased from Sigma Aldrich. Carbon and nitrogen sources were autoclaved separately to avoid Maillard reactions and formation of inhibitory compounds, and mixed when the temperature had decreased to approximately 50°C. Some ingredients such as ethanol, deoxycholate, Na₂CO₃ and HCl were added after autoclaving. Solute concentrations were made in a weight to weight percent basis.

Phenotypic profiling

In order to maximize throughput and reproducibility, a high-density array robot (Singer ROTOR HDA, Singer Instruments, Roadwater, Somerset, UK) was used to produce and replicate the selected yeast strains on the different test media. More specifically, the two 96-well plates containing the 171 stored isolates were first thawed, spotted on YGC agar (2.0% glucose, 0.5% yeast extract, 2.0% agar and 0.01% chloramphenicol) and incubated at 24°C for 2 days. Next, 96-well plates containing 150 µl YMB (YM broth) (1.0% glucose, 0.5% peptone, 0.3% malt extract and 0.3% yeast extract) in each well were inoculated with the strains using the robot and incubated overnight at 24°C on a microplate shaking platform (Heidolph, Germany) at 900 rpm. Then, the optical density at 600 nm (OD₆₀₀) of all wells was measured using a microplate reader (Molecular Devices), obtaining values ranging from 0.9 to 1.9. Subsequently, cell density was manually adjusted to OD₆₀₀ ≈ 1.0 in a second 96-well microtiter plate using sterile deionized water. This plate was used as the source plate for spotting the test media with the HDA rotor. Additionally, for a subset of 52 *M. reukauffii* and 52 *M. gruessii* randomly picked strains (Table S3) mixed cultures were prepared by inoculating the strains from two YGC

agar plates in one YMB medium plate using the HDA rotor, resulting in a co-culture of two strains. Previously, the reliability of this method to transfer equal amounts from each strain into liquid medium had been verified by counting the number of viable cells for each species (methylene blue was added) immediately after transfer using a cell chamber and magnification at 40× under the microscope. Following overnight incubation at 24°C, the OD₆₀₀ of the mixed cultures was measured and brought to 1.0 before spotting on the test media. As above, we tested if the mixture still contained comparable amounts of both species after this short incubation for 10 combinations by counting the number of viable cells for each species under the microscope. For this experiment, two parameters were discarded due to insufficient growth and therefore difficulties to accurately measure growth area (deoxycholate and NaCl 10%), so that in the end 45 parameters were analysed (see Table S2). After spotting, all agar plates were sealed using parafilm (Pechiney Plastic Packaging Company) and incubated. All tests were conducted at 24°C. After 2 days of incubation, all plates were scanned using a high-definition scanner (Seiko Epson, Nagano, Japan) (see Fig. S2a for a graphic summary of these lab procedures). To evaluate osmotolerance, plates were incubated for 12 days and regularly scanned (after 2, 4 and 12 days).

To obtain the phenotypic profiles of the strains, the colony area of each strain on each solid test plate was measured. Scanned images of the test plates were processed using ImageJ (Abramoff *et al.*, 2004), combined with the ScreenMill software especially developed for this purpose (Dittmar *et al.*, 2010). The relative growth after 2 days was calculated as the growth in a certain condition ('test condition') relative to the growth on the control medium plate for carbon and nitrogen sources. No growth was observed for the YNB negative reference plates. For all tests, strains with less than 100 pixels as growth area in the positive reference YNB–glucose plate after 2 days of incubation were discarded from the analysis. To evaluate osmotolerance, the growth rate was calculated as $yield = (\ln A_t - \ln A_0) / t$, where A_0 is the initial and A_t the final colony area after growth and t is the number of days between the first and last measurement. Growth was assessed after $t = 10$ days for sucrose and $t = 8$ days for glucose and fructose. This difference is due to the fact that the isolates showed measurable growth in sucrose in the first screening ($t = 2$ days), but they showed a delayed growth in the monomers (with measurable growth at $t = 4$ days). As resistance to inhibitors is independent from reference plates (data not shown), here the actual growth was recorded. For casein hydrolysis, the clearing zone around colonies was scored by conducting integrate area measurement using ImageJ on binary images, whose background and colony area pixel values were set to 0.

Growth experiments in liquid media

Finally, we investigated in more detail the growth of *M. reukaufii* and *M. gruessii* under different environmental conditions mimicking natural nectar characteristics (see Pozo *et al.*, 2014 for original data and references) and compared their performance between pure and mixed cultures. To that end, two strains were selected for each yeast species. These

strains originated from a single host plant and individual flower (*A. baetica*), and experiments were performed in series of two independent replicates. Briefly, cells were transferred to YMB using fresh cultures from YGC plates. YM pre-cultures were incubated with gentle agitation (280 rpm) at 24°C overnight. The next day, we inoculated a standardized amount of cells from the YM broth into the different test media (experiments performed in vials of 2 ml) (see Fig. S2b for a graphical summary of these laboratory procedures). To calculate the volume of YM broth that needed to be inoculated (V_1), we measured OD₆₀₀, referred to as OD1 in the following formula: $V_1 = (OD_2 * V_2) / OD_1$, where OD2 is the final OD we wanted to achieve in the test medium (OD₆₀₀ = 0.001, corresponding to ≈1 cell in 1 μl) and V2 is the final volume of the test medium (1 ml). The used OD2 value was based on previous experiments that were conducted in the laboratory to investigate the amount of cells that were transferred into small volumes of artificial nectar (see Canto *et al.*, 2008 for media composition) during a single visit of an insect (visit duration was established to be, on average, 10 s based on Herrera *et al.*, 2013 trials with commercial bumblebee hives). As a result, these values mimic initial cell densities in natural flowers immediately after pollinators have visited a flower and deposited yeasts in a given nectar sample. When inoculating co-cultures, we set OD2 to 0.0005 for each strain, in order to keep the same total number of cells in different experiments. These adjustments were carried out in order to ensure comparable oxygen and nutrient availability for different treatments. This difference in initial concentration of cells between single and co-cultures was corrected at computing the number of cells per μL by adding 2 as a dilution factor in the co-cultures. As a result, single and mixed treatments were comparable for each species in the analyses. For both yeast species, we previously showed that OD₆₀₀ was proportional to cell number during the log phase, or phase of balanced growth. The vials were incubated in a shaking platform (280 rpm) for 2 days at 24°C. After 2 days, growth of *Metschnikowia* cultures is known to have reached the stationary phase (Pozo *et al.*, unpubl. results). Performance of the two species was investigated in a number of test media, containing either 30% of sucrose, fructose and glucose separately, supplemented with 1% yeast extract. In addition, the performance of both species was tested in sucrose 20% plus 1% yeast extract combined with the following parameters: (i) plant secondary compounds, by adding Digitonin 8 ppm; (ii) low pH (pH 3.5, by adding HCl to the media after autoclaving); and (iii) high pH (pH 8.5, by adding CaCO₃ to the media). After incubation, 50 μl of lactophenol cotton blue was added to each vial in order to stain the cells and prevent additional growth. Samples were kept in the refrigerator, then vortexed and placed in a Neubauer counting chamber in order to be examined under the microscope at 40× (see details in Herrera *et al.*, 2009).

Data analysis

To test whether co-occurrence of yeast species at the regional scale was related to the evolutionary history of the plant species, yeast co-occurrence was modelled as a binary variable and related to the phylogenetic affiliation of the host plants using a generalized linear model (proc GLIMMIX in

SAS), in which plant families and plant species were treated as fixed factors in separate analyses, and individual nectar samples were treated as random independent sampling units. To depict evolutionary relationships between the 63 plant species studied and yeast co-occurrence, a phylogenetic tree was constructed using Phylomatic (Webb and Donoghue, 2005), with R20120829 (for plants) as reference tree. Branch lengths were computed using the 'bladj' option in Phylocom (Webb *et al.*, 2008), taking ages from Wikström and colleagues (2001). The resulting tree was then modified using R 'phytools' package to incorporate information on the relative proportion of samples containing either *M. reukaufii*, *M. gruessii* or both species (Revell, 2012). We also performed an analysis in which the strength of the phylogenetic signal (D) was determined (Fritz and Purvis, 2010) for the binary trait of co-occurrence of the two yeast species in the same nectar sample as well as in the same plant species but not in the same nectar sample. These analyses indicate if these traits are 'clumped' or randomly distributed in the phylogeny. D values that are negative or close to 0 are more phylogenetically conserved (or clumped), which can indicate non-independent evolutionary events, whereas D values closer to 1 are overdispersed and therefore can be a sign of randomness in the trait's distribution within a phylogeny. To that end, the aforementioned phylogeny was considered as reference using the function 'phylo.d' as implemented in the R package 'caper' (Orme, 2013). We also computed the phylogenetic signal by permutation ($n = 1000$) on the same tree using the relative proportions of nectar samples containing the two species using the 'phylosig' function in the R package 'phytools', which computes the K statistic (Blomberg *et al.*, 2003) and its significance by the randomization test of Blomberg (Revell, 2012).

NMDS based on Bray–Curtis dissimilarities was used to visualize patterns in the yeast community and to explore the role of host plant species in determining phenotypic profiles. The impact of yeast species (*M. reukaufii* and *M. gruessii*) on the overall phenotypic profile of the strains was tested using the 'adonis' [multivariate analysis of variance (ANOVA) based on dissimilarity matrices] function in the R package 'vegan' (Oksanen *et al.*, 2013). In this analysis, the impact of host plant origin was treated as random effect and analyses were conducted on either all tests combined, or by compound family (see Table S2): carbon sources, nitrogen sources and inhibitors. Dissimilarity matrices were calculated based on the Bray–Curtis distance using 'vegdist' functions as implemented in 'vegan'. However, in the particular case of the low water activity compound family, we used Gower distances, due to the fact that growth was scored as a rate (see above). Non-parametric Wilcoxon and Kruskal–Wallis tests were used to compare the response of each strain to each test condition, as most of the data were not normally distributed. A Bonferroni-adjusted significance level of 0.0011 (0.05/47 tests) was used to account for the increased possibility of type I error. We also calculated the coefficient of variation (CV) among strains to test whether the overall phenotypic variation differed between the two species. Following Liti and colleagues (2009), we used a Wilcoxon signed-rank test to check whether the coefficients of variation calculated for each test differed between the two species.

To test the performance of single versus mixed cultures of the two species on the solid test plates, growth rates were compared between single and mixed cultures using an ANOVA within the GLIMMIX procedure of SAS. Growth (see calculations above for solid plates) was set as the dependent variable in the models, and treatments were defined as single culture of *M. reukaufii*, single culture of *M. gruessii* and mixed culture of the two. Treatment mean differences were separated using the PDIFF option in the LSMEANS statement, where $P \leq 0.05$ was required for significance. We compared growth of the two species between pure and mixed cultures. The fact that 52 different isolates were analysed from each yeast species was considered in the analyses by declaring isolate as a random factor. In our co-culture trials, we considered interactions between species to be negative when the two species performed better in single cultures than in the mixed cultures. In contrast, in order to assign a positive effect to the interaction of the two species, we consider potentially synergistic interactions when the growth of the two species was significantly lower in the single culture treatments than in the mixed culture.

For the nectar-mimicking tests in liquid media, we did a mixed model as implemented in the SAS procedure GLIMMIX for each parameter ($n = 6$ conditions tested, see above), with growth (cells μL^{-1} that were obtained after 2 days) plus 1 as a dependent variable. Different counts per replicate in the Neubauer chamber were treated as random independent units for the analyses. The hierarchical nature of the data was declared by considering yeast isolate, replicate within yeast isolate and cell count per replicate, and yeast isolate as random effects. Cell count data for all parameters were not normally distributed. As a result, lognormal was set as distribution, with identity as link. As above, treatment was the dependent variable and treatments were defined as single culture of *M. reukaufii*, single culture of *M. gruessii*, and mixed culture of the two. Treatment mean differences were separated using the PDIFF option in the LSMEANS statement, where $P \leq 0.05$ was required for significance.

All statistical tests were conducted in R version 3.1.1 (The R Foundation for Statistical Computing) and SAS version 9.1.3 (SAS).

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Data accessibility

All data are included in the manuscript and supporting information.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Trait variance in *M. guessii* (black bars) and *M. reukaufii* (white bars) (in logarithmic scale for upper panel). Trait variance within *M. reukaufii* and *M. guessii* was calculated for each trait separately and a mean over all strains ($n = 78$ and 93 , respectively) is displayed. Tests were shown separately according to degree of variance.

Fig. S2. Experimental procedures used to test coexistence at the local scale.

Table S1. Overview of the origin of the yeast isolates used in this study.

Table S2. List of parameters tested and their concentration.

Table S3. Randomly picked isolates from EBD CSIC collection used in co-cultures.

Table S4. Significant differences in microbial growth between individual strains of each yeast species (*M. reukaufii* and *M. guessii*) and their co-culture.