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NOTES AND SHORT COMMUNICATIONS

Flowers as Islands: Spatial Distribution of Nectar-Inhabiting Microfungi among Plants of *Mimulus aurantiacus*, a Hummingbird-Pollinated Shrub

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Abstract Microfungi that inhabit floral nectar offer unique opportunities for the study of microbial distribution and the role that dispersal limitation may play in generating distribution patterns. Flowers are well-replicated habitat islands, among which the microbes disperse via pollinators. This metapopulation system allows for investigation of microbial distribution at multiple spatial scales. We examined the distribution of the yeast, *Metschnikowia reukaufii*, and other fungal species found in the floral nectar of the sticky monkey flower, *Mimulus aurantiacus*, a hummingbird-pollinated shrub, at a California site. We found that the frequency of nectar-inhabiting microfungi on a given host plant was not significantly correlated with light availability, nectar volume, or the percent cover of *M. aurantiacus* around the plant, but was significantly correlated with the location of the host plant and loosely correlated with the density of flowers on the plant. These results suggest that dispersal limitation caused by spatially nonrandom foraging by pollinators may be a primary factor driving the observed distribution pattern.

Introduction

Until recently, the prevailing theory on microbial distribution was that "everything is everywhere, but, the environment selects" [2, 6, 20]. Small propagules and large populations of microorganisms were thought to facilitate unlimited dispersal, promoting microbial ubiquity wherever the environment was suitable. Although some studies corroborated this theory [15, 18–21], others found patterns inconsistent with this theory at large [48] and small scales [39] as well as through time [3, 29, 36, 47]. With these findings, many authors now regard dispersal limitation—the situation in which a species' limited capability for dispersal prevents it from reaching areas of suitable habitat [10]—as a potentially important factor influencing microbial distribution [41, 47, 48]. However, microbial dispersal is hard to trace, and microbial habitat requirements are often unknown, making it difficult to decouple effects of environmental factors from those of dispersal limitation [29].

Species of yeast and other microfungi found in floral nectar offer unique opportunities for the study of microbial distribution and dispersal limitation. Floral nectar is initially sterile and microfungi disperse to flowers on bees, birds, and other pollinators [14], whose movement is more readily traceable than that of the microbes themselves. Flowers are discrete, island-like habitats and microbial metapopulations can be analyzed at multiple scales within and between plants, which function as microbial "archipelagos." Moreover, only a small number of species can inhabit floral nectar due to high sugar content (about 20–50%) [26, 27] and antimicrobial compounds [35], making them relatively simple to analyze compared with other microbial communities [11, 35]. Despite these advantages of nectar-inhabiting microfungi as a study system, little is known about their distribution patterns or the role that dispersal limitation may play in generating the patterns.

In this paper, we investigate distribution patterns of nectar-inhabiting microfungi in the flowers of *Mimulus aurantiacus*, or sticky monkey flower, in central California. We hypothesize that nectar-inhabiting microfungi show distinct distribution patterns possibly attributable to spatially nonrandom

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dispersal by pollinators, which are thought to be the main dispersal agent [9]. At our study site, Anna's hummingbird (*Calypte anna*) is the main pollinator of *M. aurantiacus* flowers, although Allen's hummingbird (*Selasphorus sasin*), Rufous hummingbird (*Selasphorus rufus*), and occasionally bees (*Bombus vosnesenskii* and *Xylocopa micans*) have also been noted visiting *M. aurantiacus* flowers. Since visits by insect pollinators are not common [45], this study focuses on spatial variation in environmental and floral factors that might influence nectar availability and hummingbird movement, including light intensity, flower density, neighboring plant density, and nectar volume per flower [7, 44].

Methods

Study Site

The survey was conducted in a 0.25-km^2 area at the Jasper Ridge Biological Preserve (JRBP) on the San Francisco peninsula of California. This area contains several vegetation types, including chaparral, open woodland, and broadleaf evergreen forest within a relatively small area (Fig. S1). Our preliminary survey at JRBP detected nectar-inhabiting microfungi in multiple plant species, including *M. aurantiacus*, *Lepechinia calycina* (pitcher sage, Lamiaceae), *Pedicularis densiflora* (Indian warrior, Orobanchaceae) and *Eriodictyon californicum* (yerba santa, Boraginaceae). In this study, we focus on *M. aurantiacus*, a common species at JRBP, to standardize host species identity. This species is a shrub native to California and Oregon. In JRBP, it is found under a range of conditions, from relatively moist oak woodland to dry, open chaparral (Fig. S1), and blooms from approximately late March to early July [37].

Nectar Sampling

For this study, 16 *M. aurantiacus* plants under varying microenvironmental conditions were chosen for sampling of nectar. Flowers from each plant were collected in June 2010, which coincided with the height of *M. aurantiacus* flowering activity at JRBP. Flower age at the time of collection was standardized to 6 days by marking flowering buds before they were open and recording the day of first flower opening. Although individual *M. aurantiacus* flowers bloom for up to 10 days in the field, 6 days was chosen as the time to harvest the flowers because our preliminary survey indicated that 6 days would provide a reasonable amount of time for yeast to disperse to the flower while reducing the number of flowers lost to wilting [42]. Six to 12 flowers were sampled per plant, depending on the number of flowers produced by the plant at the time of sampling. A total of 192 flowers were sampled.

Immediately after harvesting each flower, nectar was extracted with a 5-μl microcapillary tube, volume measured, diluted in 60 μl of distilled H_2O, and stored at 4°C until being processed within 1 week of nectar collection in the field. Dilution plating was used to estimate the density of colony forming units (CFUs) in each sample. Briefly, each sample was further diluted in distilled H_2O and the nectar–water solution spread on yeast extract–malt extract agar (YM agar) plates [49] using a sterile spreading rod. Resulting colonies were counted from plates with dilutions yielding approximately 100 CFUs after 2 days of incubation at 25°C. From each plate, up to 12 colonies were randomly chosen for separate DNA extractions (see below). The density of yeast per collected flower was estimated using the dilution factor and the number of colonies recovered after plating. Although this method disregards species that may be unculturable, it is commonly used in similar studies and has been found to accurately represent the species composition and cell density of nectar-inhabiting microfungi [9].

Hummingbird Sampling

Two experiments were carried out to ascertain that hummingbirds were dispersal agents for microfungi to *M. aurantiacus* flowers. In the first, hummingbirds were captured by mist-netting, and their tongues and beaks assayed for the presence of microfungi. To this end, after capture, hummingbirds were fed initially sterile sugar water. The remaining water, which had come into contact with birds' beaks and tongues, was plated on YM agar, and the resulting colonies were DNA-sequenced for species identification. In the second experiment, we caged *M. aurantiacus* plants to experimentally deny access by hummingbirds. Because the mesh size of the cages was large enough to allow access by potential insect pollinators, but not hummingbirds, we were able to evaluate the role of hummingbirds for dispersal of microfungi through comparison of flowers in vs. outside the cages. Yeast abundance in nectar was surveyed by harvesting flowers for nectar sampling when they were 5 days old.

Molecular Methods

Fungal DNA was extracted and amplified using the Sigma Extract-N-Amp tissue polymerase chain reaction (PCR) kit (Sigma-Aldrich, Inc., Saint Louis, MO, USA). PCR reactions were performed in a 20 μl volume using 0.8 μl of extracted DNA, 10 μl of Extract-N-Amp PCR ReadyMix

(Sigma-Aldrich, Inc., Saint Louis, MO, USA), 0.15 μl of each primer at 50 μM and 8.9 μl of H_2O. Amplification was performed using the D1/D2 domains of the large subunit nuclear ribosomal RNA with the primers NL1 (5′-GCA TATCAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG GAC GG-3′) [38], which are commonly used for yeast identification [9, 27, 32, 34].

PCR amplification was conducted using a touchdown PCR protocol with the following settings: an initial denaturation step of 94°C for 3 min, denaturation at 94°C for 30 s, 10 cycles decreasing in 0.5°C increments from 56.5°C to 51.5°C for 30 s each, 20 cycles of 51.5°C for 45 s followed by 72°C for 45 s, and a final elongation step of 72°C for 10 min. PCR products were separated by gel electrophoresis using 1.25% sodium boric acid gel [8] and visualized using ethidium bromide staining and subsequent UV transillumination (Fotodyne Inc., Hartland, WI, USA). Samples that produced a visible band during gel electrophoresis were sequenced by Elim Biopharm (Hayward, CA, USA), using an ABI 3730 XL automatic DNA sequencer.

Sequence Analysis

Forward and reverse sequences were aligned using Clustal W (1.83) software. The consensus sequences were grouped into operational taxonomic units (OTUs) using Geneious Pro bioinformatics software (Biomatters Ltd., Auckland, New Zealand). OTUs were defined as groups of sequences sharing 98% pairwise similarity [32]. A representative sequence of each OTU was used to perform Basic Local Alignment Search Tool (BLAST) searches against the National Center for Biotechnology Information's GenBank. Representative sequences were also placed into a most likely tree using sequences from other studies of nectar yeast and a recent phylogeny of the Saccarhomycetales [46]. All sequences were aligned in Geneious using the MAFFT [31] plugin and the most likely tree estimated using PhyMyl [25]. Species names were assigned using a combination of BLAST match and phylogenetic placement (Fig. S2, Table 1).

Environmental Factors: Flower Density, Neighboring Plant Density, and Light Availability

The number of flowers on each sampled plant was recorded at the time of flower collection. The percent cover of *M. aurantiacus* plants within a 3-m radius of each sampled plant was also recorded to estimate neighboring plant density. The amount of photosynthetically active radiation transmitted through gaps in the overlying tree canopy (molecules per square meter per day) was estimated for each of the 16 plants for the year December 2009–December 2010 [1, 12], using photographs taken in December 2009 with a digital SLP camera with circular fish eye lens and the Gap Light Analyzer (Simon Fraser University, Institute of Ecosystem Studies, 1999).

Statistical Analyses

To determine which environmental factors were correlated with microfungal distribution, we performed linear regressions using nectar volume, light intensity, and flower density as predictors of the percentage of flowers per plant occupied by microfungi using JMP software v. 8.02 (SAS Institute Inc., Carey, NC, USA). We also performed the same analysis to predict the percentage of flowers per plant occupied by the most common microfungal species in our study, *Metschnikowia reukaufii*. Other species were too rare to analyze individually. In addition, we created pairwise similarity matrices of geographic distances between plants, differences in flower density, and the percent of flowers from which microfungi were detected on each plant. Statistical significance of correlations between these variables was tested using Mantel and partial Mantel tests in the programming environment R version 2.7.2 (R Core Development Team 2008) and using the packages Ecodist [24] and Vegan [40].

Results

Microfungi were detected from 15 of the 16 plants sampled, with up to nine species per plant (Fig. 1a) and from 54 of the 97 unwilted flowers sampled, with an average of 4,960 CFUs (min=19.2, max=94,480) per microliter of nectar in samples that contained microfungi. Across the study, we observed a total of nine species using a 98% sequence similarity cutoff and 16 species using a 99% cutoff (Table 1). However, the observed number of species per flower was low, with 97% of colonized flowers having only one fungal species (Fig. 1b, c).

The proportion of flowers per plant colonized by microfungi was not significantly correlated with flower density (r^2=0.10, p=0.22), neighboring plant density (r^2=0.037, p=0.59, Fig. 2b), light availability (r^2=0.06, p=0.36), or average nectar volume per flower (r^2=0.18, p=0.09). However, the relationship between microfungal frequency and flower density was significant when one outlier (a small plant with high flower density but low flower number) was excluded (r^2=0.35, p=0.01; Fig. 3).

Table 1 Taxonomic assignments of microfungi observed in this study

Species identity	Number of sequences from nectar	Number of sequences from birds	Top BLAST match (accession number)	% match	Accession number
Aspergillus fumigatus	0	1	AY660917	100	JN642540
Auerobasidium pullulans	3	2	GQ911488	99.8	JN642535
Beauveria bassiana	1	1	AY283555	99.6	JF906819
Candida albicans	0	3	FJ627953	99.7	JN652537
Candida parapsilosis	0	8	EU660860	100	JN642532
Candida quercitrusa	0	2	DQ466526	100	JN642539
Candida rancensis	20	11	EU523604	100	JN642531
Collophora rubra	0	1	HQ433106	96	JN642541
Cryptococcus albidosimilis	0	1	GU460168	100	JN642543
Cryptococcus flavescens	0	1	AM748548	100	JN642542
Cryptococcus sp.	1	1	DQ513279	100	JF906824
Hanseniaspora uvarum	0	1	EU268654	100	JN642546
Hanseniaspora valbyensis	14	11	U73596	99.8	JG906826
Metschnikowia gruessii	3	0	AF406913	99.8	JF906827
Metschnikowia koreensis	4	0	AB617390	99.5	JN642536
Metschnikowia kunwiensis	0	8	JF809869	100	JN642533
Metschnikowia reukaufii	166	0	JF809868	100	JN642529
Metschnikowia sp.	0	8	JF809868	92.1	JN642530
Penicillium toxacarium	0	1	EF198659	100	JN642544
Pichia fermentans	0	1	EF554827	99.8	JN642545
Rhodotorula sp.	0	3	AF387138	100	JN642538
Starmerella bombicola	2	4	HQ111047	99.7	JN642534
Total	214	69			

A highly significant positive correlation was found between the spatial proximity of plants and the frequency of microfungal colonization (Mantel r=0.32, p=0.008; Fig. 4) and *M. reukaufii* colonization (Mantel r=0.467, p=0.001; Figs. 2a and 4). Although flower density showed spatial autocorrelation when the outlier plant was removed (r^2=0.319, p=0.003), a partial Mantel test indicated that the positive correlation between host plant spatial proximity and microfungal colonization frequency was significantly positive even after the effect of flower density was controlled for (Mantel r=0.32, p=0.008).

The number of flowers analyzed from a given plant was not significantly correlated with either the percent of flowers found with yeast (r^2=0.095, p=0.13), the percent

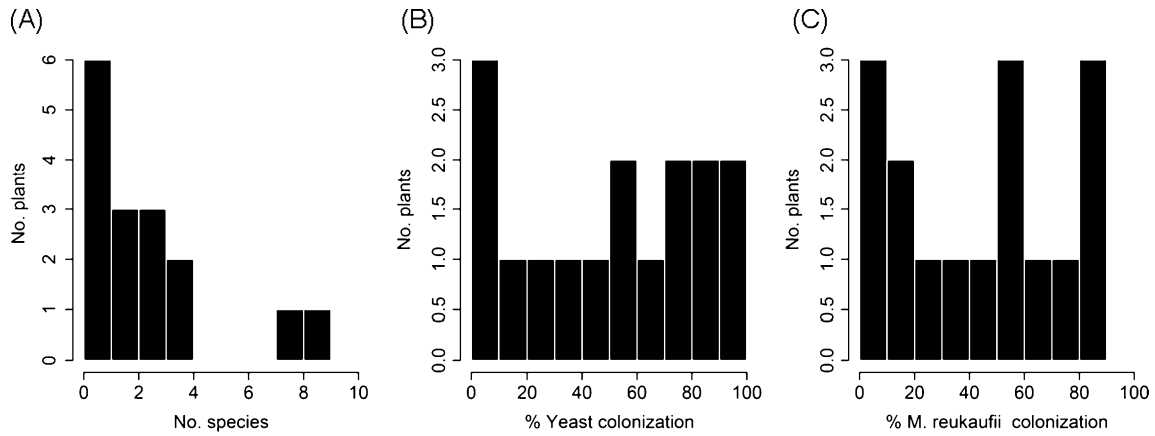

Figure 1 Histograms summarizing **a** the number of yeast species detected per plant, **b** the frequency of yeast colonization (% of flowers from which yeast was detected), and **c** the frequency of colonization by the most commonly detected species, *M. reukaufii* (% of flowers from which *M. reukaufii* was detected) in individual plants of *M. aurantiacus*

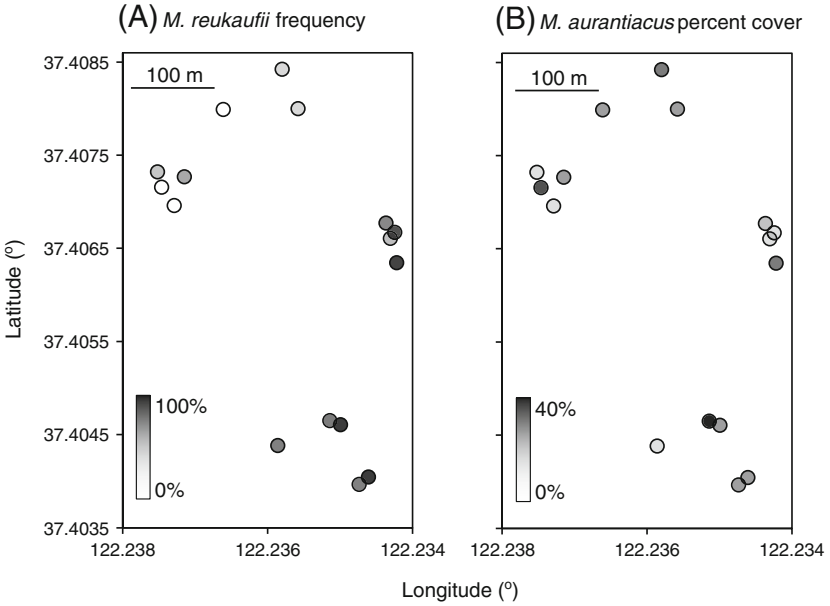

Figure 2 Graphical representation of **a** flower colonization by the yeast *M. reukaufii* per plant and **b** estimated percent cover of *M. aurantiacus* plants surrounding each plant from which nectar was sampled. GPS coordinates are plotted along the *X*- and *Y*-axis with points representing individual plants according to latitude and longitude. Points are shaded from *white* to *black*, with *white* indicating 0% flowers colonized and *black* indicating 100% flowers colonized in **a**, and *white* indicating 0% estimated percent cover and *black* indicating 40% estimated percent cover by *M. aurantiacus* plants in a 3 m radius of each sampled plant in **b**

of flowers found with the predominant species, *M. reukaufii* (r^2=0.023, p=0.26) or the number of yeast species found per flower (r^2=0.084, p=0.15), indicating that sampling bias did not affect our results.

We captured over 30 hummingbird (*C. anna*) individuals by mist-netting within the study area and sampled the bills of approximately ten birds. The results confirmed that hummingbirds that visited *M. auranticus* flowers carried viable cells of some of the same species of microfungi as found in *M. aurantiacus* nectar (Table 1). In the caging experiment, microbial abundance in nectar was significantly higher in noncaged flowers than in caged flowers (Fig. S3). Further, floral nectar was more likely to contain microbes in noncaged flowers than in caged flowers (number of flowers from which microbes were detected: 22 of 36 noncaged flowers vs. 13 of 36 caged flowers; Pearson's chi-square test, p<0.05).

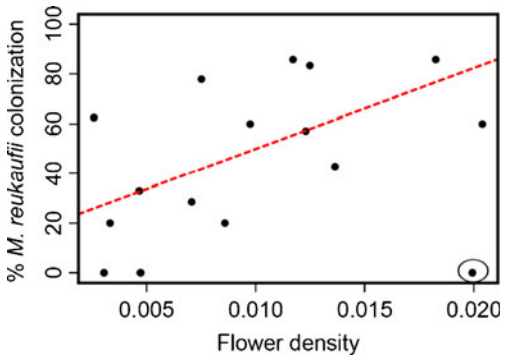

Figure 3 Relationship between the percentage of flowers colonized by *M. reukaufii* and the density of flowers (number of flowers/cm^2 of estimated surface volume of plant). Flower density was significantly correlated with *M. reaukaufii* presence when one outlier (circled) was removed (r^2=0.354, p=0.01). However, with the outlier included in the analysis, flower density was not significantly correlated (r^2=0.101, p=0.22)

Discussion

We found nonrandom distribution patterns in nectar-inhabiting microfungi (Fig. 4) with distinct areas of high and low prevalence within the study area (Fig. 2a). Several environmental and floral factors measured, including light intensity, nectar volume, flower density, and neighboring plant density, failed to explain these patterns. The only factor of marginal significance was flower density, which became statistically significant when an outlier was omitted. Spatially nonrandom foraging of pollinators in response to flower density may have caused dispersal limitation in the microfungi they carried. However, microfungal distribution remained nonrandom even after the effect of flower density was taken into consideration. This pattern may be attributable to spatially nonrandom foraging by pollinators that was affected by factors other than the environmental and floral variables we examined.

One such factor may be "trap-lining" by hummingbirds [30]. Hummingbirds often visit the same clumps of flowers in a regular, sequential fashion, a behavior that reduces energy expenditure and increases nectar reward as hummingbirds can track which flowers they have last visited [22, 44]. Along with trap-lining, territoriality ensures that most flowers in a clump will be visited [44].

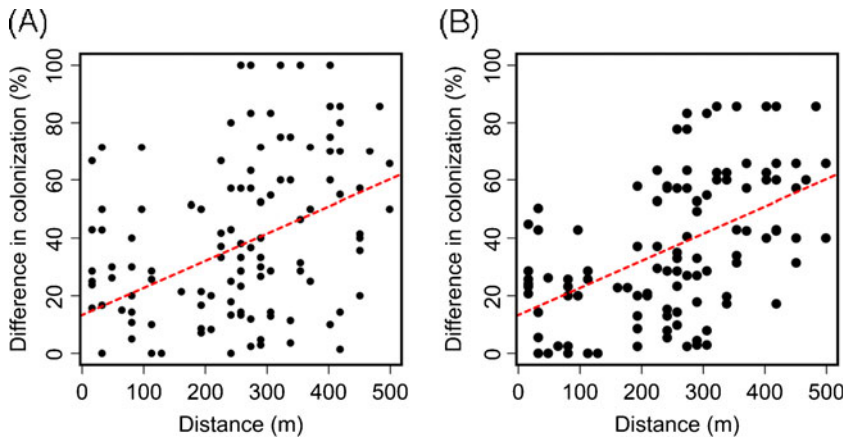

Figure 4 **a** Correlation between the percentage of flowers colonized by yeast per plant and the distance between host plants (Mantel r=0.319, p=0.008) and **b** correlation between the percentage of flowers colonized by *M. reukaufii* per plant and the distance between host plants (Mantel r=0.467, p=0.001). Matrices used for the Mantel tests were similarity matrices of distance in meters between pairs of plants and pairwise differences in the percent yeast colonization

It seems feasible that trap-lining and territoriality result in heavily fungal-inoculated clumps of flowers in some parts of a given area but not in other parts. As Anna's hummingbird is the main visitor of *M. aurantiacus* flowers [16, 17, 45], their behavior may have created the observed spatial patterns. A study conducted at JRBP found that Anna's hummingbird's males aggressively defend their territories [13]. The prime vegetation for male territories is flowering chaparral [13], where *M. aurantiacus* are in highest densities (Fig. S1). The core territory of each male was approximately 1,000 m^2 in size, and 16 males occupied territories in an area the size of approximately 1.7 km^2, whereas females were nonterritorial and nest in woodlands, feeding on flowers wherever possible [13].

Several microfungal species (e.g., *Candida rancensis*, *Hanseniaspora valbyensis*, *Starmerella bombicola*) were detected from both nectar and hummingbird samples (Table 1), indicating that hummingbirds were indeed their dispersal agent. Although *M. reukaufii* was not detected from hummingbirds, the sample size was somewhat small (ten birds) and the sampling took place earlier than the time of the nectar sampling. Further research on hummingbird behavior along with more microfungal sampling from hummingbirds is needed to directly test for a connection between nectar-inhabiting microfungi and pollinator movement.

In this study, we measured nectar quantity, but not quality, which may also have affected microfungal distribution. Varying concentrations of amino acids and sugars in nectar are known to affect pollinator visitation [4, 5]. In addition, some yeast species may affect the concentrations of amino acids and sugars, thus potentially affecting pollinator visitation as well as the growth of late-arriving yeast species [26, 42]. Nectar chemistry may provide a strong environmental filter for some microfungal species [28]. However, variation in nectar chemistry between plants may not always limit colonization of a general nectivorous species like *M. reukaufii*. Laboratory studies indicate that nectivorous fungal species isolated from *M. aurantiacus* plants at JRBP can grow on different sugar sources, indicating a broad range of tolerance [42].

Nectar-inhabiting microfungi in Europe, South Africa, and elsewhere seem to be characterized by a similarly low level of species diversity, with *M. reukaufii* being one of the dominant species [9, 14, 27, 28, 33, 43]. These studies, combined with our results, indicate that nectar-inhabiting microfungal communities in geographically distant locations may consist of similar species, suggesting the possibility that nectar-inhabiting microfungi have a high capacity for long-distance dispersal, yet show dispersal limitation within local areas. Sampling more plants in larger areas should provide a more comprehensive understanding of their distribution patterns.

One potential reason for the widespread dominance of *M. reukaufii* is that this species is superior to others in local competition. This reason seems unlikely, however, as a laboratory study has suggested that other species, namely *C. rancensis* and *M. koreensis*, may be as competitive as *M. reukaufii* and can completely exclude it if they arrive earlier [42]. Thus, mechanisms other than local interactions within flowers may be necessary to explain the distribution patterns we found. Differences between species in dispersal ability, if they exist, may be one such explanation.

Along with findings from previous work [9, 14, 23, 27, 28, 44], the nonrandom distribution patterns we have reported in this paper reinforce the prospect of these microfungal species as a useful model system for understanding the role of dispersal in determining microbial distribution. Future research should more directly investigate dispersal limitation through both observations and experiments.

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