RESEARCH ARTICLE

Nonrandom genotype distribution among floral hosts contributes to local and regional genetic diversity in the nectar–living yeast *Metschnikowia reukaufii*

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Abstract

Environmental heterogeneity has been often suggested as a major driving force preserving genetic variation in clonal microorganisms. This study examines this hypothesis for the specialized nectar-dwelling, clonal yeast *Metschnikowia reukaufii* (*Ascomycota*, *Saccharomycetales*). We examined whether *M. reukaufii* subpopulations associated with flowers of different host plant species, and different individuals of the same host species, differed in genetic characteristics. Amplified fragment length polymorphisms (AFLP) fingerprints of *M. reukaufii* strains isolated from floral nectar of different host species and individuals sampled at different spatial scales revealed a strong host-mediated component of genetic and genotypic diversity at all scales considered. Genotypes were nonrandomly distributed among flowers of different species and, in the case of the single host species studied in detail (*Helleborus foetidus*), also among flowers of conspecific individuals coexisting locally. These host-mediated patterns of genetic structuring are compatible with those expected under the diversifying selection hypothesis for the maintenance of local and regional genetic diversity in clonal organisms. It is proposed that a combination of intrafloral selection and biased pollinator-mediated migration may ultimately account for observed host-mediated genetic structuring in populations of *M. reukaufii*.

Introduction

Patterns of genetic diversity have been investigated in countless plant and animal populations, but infrequently among other eukaryotic organisms. This singularly applies to free-living unicellular eukaryotes such as protists or microfungi (termed yeasts hereafter), for which only a handful of studies have so far described the genetic structure of their wild populations (Koufopanou *et al.*, 2006; Lachance *et al.*, 2008; Wardlaw *et al.*, 2009; Herrera *et al.*, 2011; Rengefors *et al.*, 2012). In the case of yeasts, certain biological features may give rise to atypical patterns of genetic structure and diversity. For example, asexuality is often the prevailing or exclusive reproductive mode in wild yeasts (Wardlaw *et al.*, 2009; Zeyl, 2009; Herrera *et al.*, 2011). Theoretical models predict that clonal organisms should exhibit reduced genotypic diversity (Balloux *et al.*, 2003; Bengtsson, 2003), a prediction frequently upheld by empirical observations (Delmotte *et al.*, 2002; Halkett *et al.*, 2005). Nevertheless, populations of nectar-living clonal yeasts may harbor a considerable wealth of genetic and genotypic diversity (Herrera *et al.*, 2011), a pattern with potential evolutionary significance that demands explanation. Diversifying selection (Ellstrand & Roose, 1987; Kassen & Rainey, 2004) has been hypothesized to contribute to high genetic diversity in these floricolous yeasts (Herrera *et al.*, 2011). According to this hypothesis, intra- and interspecific variation in floral nectar features ordinarily occurring in natural plant communities (e.g. Petanidou, 2005; Nicolson & Thornburg, 2007) should eventually give rise to a host-mediated component of population-wide genetic diversity. In this way, nonrandom genotype distribution among host flowers would contribute to maintain high genetic and genotypic diversity in the face of prevailing or exclusive clonality (Herrera *et al.*, 2011).

This study addresses the following questions for the clonal nectar-dwelling yeast *Metschnikowia reukaufii* Pitt

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and M. W. Miller (*Ascomycota*, *Saccharomycetales*): (1) At the regional level, do host plant species differ in the genetic characteristics of *M. reukaufii* yeasts living in their floral nectars?; (2) At the local plant community level, does the nectar of coexisting, simultaneously blooming hosts of different species harbor genetically distinct *M. reukaufii* subpopulations?; and (3) Within a local host plant population, do conspecific individuals differ in the genetic characteristics of associated *M. reukaufii* yeasts? These questions will be examined by analyzing amplified fragment length polymorphisms (AFLP) fingerprints of *M. reukaufii* strains isolated from floral nectar of different host species and individuals sampled at various spatial scales.

Materials and methods

Study system

Differences between host plant species and individuals in genetic characteristics of associated *M. reukaufii* yeasts were investigated in the Sierra de Cazorla region, a well-preserved natural area in Jaén province, southeastern Spain. *Metschnikowia reukaufii* is an extreme microhabitat specialist found always in association with flowers and floral nectar (Lachance *et al.*, 2001; Brysch-Herzberg, 2004), and it is the commonest species of nectar-dwelling yeast in our study region (Pozo *et al.*, 2011). Colonizing inocula are brought to flowers by foraging pollinators (Brysch-Herzberg, 2004; Herrera *et al.*, 2010). Vegetative cells found in floral nectar are diploid and proliferate there by multilateral budding, often reaching densities $> 10^5$ cells mm^{-3}. Under stringent culture conditions, they may produce asci and ascospores and reproduce sexually (Giménez-Jurado *et al.*, 1995), but sexual reproduction has been not observed to occur naturally in the nectar-living populations of our study area despite detailed microscopical examination of thousands of nectar samples (C. M. Herrera & M. I. Pozo, unpublished observations). In addition, analyses of multilocus linkage disequilibrium similar to those applied by Herrera *et al.* (2011) to *Metschnikowia gruessii* have revealed that population structure of *M. reukaufii* does not exhibit genetic signatures of regular recombination and that the species should be considered predominantly or exclusively clonal in the wild (C. M. Herrera, unpublished data). All isolates considered in this study were in the first place tentatively identified as *M. reukaufii* on the basis of distinctive cell and colony morphological features. All identifications were then corroborated by two-way sequencing the D1/D2 domain of the 26S ribosomal RNA gene, assembling a consensus sequence, and comparing the result with the corresponding sequence for the *M. reukaufii* type strain (CBS5834) in GenBank (Accession Number U44825).

Field sampling

Metschnikowia reukaufii populations were sampled in 2008 and 2010, following a different sampling scheme on each year. In a multihost, regional sampling, 30 *M. reukaufii* isolates were obtained in 2008 from floral nectar samples of eight plant species from seven different sites (Table 1). The plant species were chosen because prior studies had shown that *M. reukaufii* was abundant in their floral nectars (Pozo *et al.*, 2011). Sampling localities encompassed an area of *c.* 2400 ha, and distances between nearest and farthest sites were 0.7 and 12 km, respectively. The collection period (May–June) corresponded to the flowering peak of the insect-pollinated plant community in the study region. The main pollinators of all species sampled were large social (bumble bees, *Bombus*) and solitary (*Anthophora*, *Eucera*, *Megachile*, *Xylocopa*) bees (Table 1). Yeast isolates were obtained from single-flower nectar samples using the methods described in detail by Pozo *et al.* (2011), which consisted basically of streaking nectar onto Yeast Malt plus chloramphenicol (YMC) agar plates and obtaining isolates from the resulting colonies following morphological criteria. Each sampled flower was taken from a different plant, and a single *M. reukaufii* isolate was obtained from each nectar sample.

A paired-host sampling scheme was undertaken in April 2010. Nectar from the two locally most abundant coflowering species was sampled at each of three different sites (Fuente Bermejo, La Cabrilla, and Rastrillos de la Víbora; FB, LC, and RV hereafter; Table 1). Distances between sites ranged between 5 and 11 km, and they were located in the same general area sampled in 2008. Local host pairs were *Helleborus foetidus* and *Narcissus cuatrecasasii* in FB and RV, and *Helleborus foetidus* and *Primula vulgaris* at LC. The number of plants sampled and *M. reukaufii* isolates obtained at each site are shown in Table 1. One single-flower nectar sample was obtained from each sampled plant, and whenever possible, five yeast isolates were obtained from every nectar sample by randomly picking up colonies from the culture obtained after streaking the nectar onto YMC agar plates. Many nectar samples from *P. vulgaris* and *N. cuatrecasasii* failed to furnish *M. reukaufii*, which explains the small number of isolates and unbalanced sample sizes for these species (Table 1). At each site, flowering individuals of the two species sampled were spatially intermingled, occurred at short distances of each other, and collectively encompassed areas of *c.* 0.63 ha (FB), *c.* 0.46 ha (LC), and *c.* 0.52 ha (RV; convex polygon area estimates). As for the plant species sampled in 2008, bumble bees and large solitary bees were also the main pollinators of the three host plant species sampled in 2010 at FB, LC, and RV (Table 1).

Table 1. Collection details for the *Metschnikowia reukaufii* isolates included in the amplified fragment length polymorphisms (AFLP) study of genetic variation across floral nectar of different host plants at two contrasting spatial scales

Sampling scheme/Host plant*	Main floral visitors	Latitude (ºN)	Longitude (ºW)	Collection date	Number of isolates
Multihost, regional sampling					
Anthyllis vulneraria (4)	LSB	37.9410	2.8343	May 15, 2008	4
Aquilegia vulgaris (4)	BB	37.8995	2.8378	May 25, 2008	4
Erinacea anthyllis (3)	BB, LSB	37.9314	2.7818	May 15, 2008	3
Iris foetidissima (3)	BB	37.9300	2.9292	June 13, 2008	3
Linaria aeruginea (4)	LSB	37.9272	2.7837	May 15, 2008	4
Linaria lilacina (4)	LSB	37.9359	2.8393	May 25, 2008	4
Marrubium supinum (4)	BB	37.9132	2.8707	June 7, 2008	4
Tetragonolobus maritimus (4)	LSB	37.8995	2.8378	May 18, 2008	4
Paired-host, local sampling					
Fuente Bermejo (FB)		37.9269	2.8396		
Helleborus foetidus (8)	BB			April 24, 2010	40
Narcissus cuatrecasasii (11)	LSB			April 20, 2010	5
La Cabrilla (LC)		37.9294	2.7820		
Helleborus foetidus (13)	BB			April 27, 2010	5
Primula vulgaris (6)	LSB			April 27, 2010	10
Rastrillos de la Víbora (RV)		37.8968	2.9010		
Helleborus foetidus (8)	BB			April 23, 2010	40
Narcissus cuatrecasasii (11)	LSB			April 23, 2010	15

*Number of distinct plant individuals sampled in parentheses.
BB, bumble bees (*Bombus*); LSB, large solitary bees (Anthophoridae, Megachilidae).

AFLP genotyping

All *M. reukaufii* isolates obtained from nectar samples ($N = 30$ and 115 isolates in 2008 and 2010, respectively) were DNA fingerprinted using the AFLP technique. This method has proven useful to reveal intraspecific genetic divergence in a variety of eukaryotic microorganisms including yeasts (e.g. Laitung *et al.*, 2004; Litvintseva *et al.*, 2006; Herrera *et al.*, 2011; Rengefors *et al.*, 2012). The AFLP analysis was performed essentially as originally described by Vos *et al.* (1995) except for the modifications required by the use of fluorescent dye-labeled selective primers. Restriction–ligation was conducted using *Pst*I/*Mse*I endonuclease mixture and double-stranded adaptors. After an initial screening of primer pair combinations, a total of four *Pst*I + 2/*Mse*I + 2 primer pairs were chosen that provided reliable, consistently scorable results, and each isolate was fingerprinted using these four combinations. Primer combinations used for fingerprinting the 2008 isolates (*Pst* AT–*Mse* CA, *Pst* AC–*Mse* CA, *Pst* AG–*Mse* CG, *Pst* AA–*Mse* CC) differed slightly from those used with the 2010 isolates (*Pst* AT–*Mse* CA, *Pst* AC–*Mse* CA, *Pst* AG–*Mse* CA, *Pst* AC–*Mse* CT), as some combinations were replaced in 2010 to improve repeatability. This difference was inconsequential to results, as fingerprints obtained with the two primer sets were analyzed separately. Fragment separation and detection was made using an ABI PRISM 3130xl DNA sequencer, and the presence–absence of each marker in each sample was scored manually by visualizing electropherograms with GENEMAPPER 3.7 software. Only fragments ≥ 150 base pairs in size were considered to reduce the potential impact of size homoplasy (Vekemans *et al.*, 2002). Intraplate repetitions were run for 20% of randomly chosen isolates, and genotyping error rates were estimated separately for each marker as the ratio of the number of mismatches to the number of replicated samples. Only markers with error rates $\leq 4\%$ were retained for analysis (76 markers in the 2008 sample and a different set of 76 markers in the 2010 sample). Mean genotyping error rate computed over all markers and primer combinations was 1.89%.

Data analysis

Genetically identical isolates belonging to the same clone could appear as different genotypes because of small differences arising from AFLP genotyping errors (Meirmans & Van Tienderen, 2004). The number of distinct genotypes occurring in a given sample was estimated using the GenoType program, which allows to enter a pairwise genetic distance threshold below which isolates are considered clonemates (Meirmans & Van Tienderen, 2004). This threshold was set to the mean genetic distance expected between identical genotypes whose AFLP fingerprints differ in a number of markers equal to the product of mean per-marker genotyping error rate (0.0189) by the number of markers scored (76). When the estimated

number of genotypes in the sample was smaller than the number of isolates (2010 samples), the GenoType program was used to assign isolates to the putative genotypes occurring in the sample.

Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was used to evaluate the magnitude and statistical significance of various sources contributing to *M. reukaufii* genetic diversity, namely host species for the 2008 regional sample; site and host species nested within site for the 2010 paired-host samples; and site and individual plant nested within site for the FB and RV 2010 samples from *H. foetidus*. Computations were conducted using the amova function in the pegas package (Paradis, 2010) for the R environment (R Development Core Team, 2010). Analyses were based on pairwise, isolate-by-isolate genetic distance matrices obtained with the dist function in the R stats package. Distance method was set to 'euclidean', which provided metric distances as required by AMOVA (Excoffier *et al.*, 1992). Φ_{ST} values, an analog of F_{ST}, were also obtained from the AMOVA analyses, and their statistical significance was determined by permutations. In hierarchical AMOVAs, the random permutation scheme followed Excoffier *et al.* (1992: Table 3).

Results

Regional sampling

The 30 *M. reukaufii* isolates obtained in 2008 from eight host plant species had unique AFLP profiles. Using the predefined threshold, the GenoType program led to an estimate of 30 distinct genotypes in the sample. Doubling the threshold did not entail any change in expected number of genotypes, and tripling it only slightly reduced number of genotypes from 30 to 29. The uniqueness of AFLP profiles in the 2008 sample thus predominantly or exclusively reflected actual genotypic differences between isolates.

There was a significant host-related component to *M. reukaufii* regional genetic diversity, as denoted by the AMOVA of genetic distances between isolates. The AMOVA -based estimate of genetic differentiation between hosts, $\Phi_{ST} = 0.216$, was significantly greater than zero ($P < 0.0001$; permutation test with 10^4 repetitions). About 21% of total genetic diversity occurring in the 2008 sample was thus accounted for by differences among host plant species in genetic characteristics of associated *M. reukaufii* isolates. Samples from different host species came from different sites (Table 1), and hence, the host-related component of *M. reukaufii* genetic diversity might actually reflect spatial genetic structuring. No significant relationship existed, however, linking pairwise genetic distance between yeast isolates from different host species (pairwise Φ) and geographical distance between the corresponding sampling sites ($r = -0.228$, $P = 0.21$, Mantel test with 10^4 repetitions). Genetic divergence between *M. reukaufii* isolates originating from flowers of *Aquilegia vulgaris* and *Tetragonolobus maritimus*, two hosts sampled from the same site (Table 1) and flowering there side by side, approached significance ($\Phi_{ST} = 0.190$, $P = 0.053$) despite the small number of isolates per host. These results support the contention that genetic differences between *M. reukaufii* from different host species were not a spurious artifact of spatial structuring, an interpretation upheld by results of paired-host sampling in 2010 shown in the next section.

Paired-host species

Sixty-one distinct AFLP profiles were present in the set of 115 *M. reukaufii* isolates from nectar of *H. foetidus*, *N. cuatrecasasii,* and *P. vulgaris* sampled in 2010 from FB, LC, and RV sites. After accounting for the possible inflation of genotype number caused by AFLP genotyping errors, the GenoType program estimated 36 distinct genotypes in the sample, 29 of which were represented by single isolates. The remaining seven genotypes accounted collectively for 86 isolates. For the three sampling sites combined, isolates belonging to the seven most frequent genotypes were nonrandomly distributed across the three host species sampled (likelihood ratio chi-square = 46.24, d.f. = 12, $P < 0.0001$).

The hierarchical AMOVA on the pairwise genetic distances matrix between 115 isolates revealed a significant genetic variance component associated with differences between coexisting host species within sites, but not between sites (Table 2). The sample-wide estimate of *M. reukaufii* genetic differentiation between coexisting hosts within the same site ($\Phi = 0.194$) revealed that, on average, about 20% of total genetic diversity represented in the sample was accounted for by small-scale, local variation between coflowering host species. The extent of *M. reukaufii* genetic differentiation between local hosts

Table 2. Hierarchical AMOVA on the pairwise matrix of genetic distances between the 115 *Metschnikowia reukaufii* isolates obtained in 2010 from nectar samples of three pairs of locally coexisting, coflowering host plants at three different sites (see Table 1 for sampling details)

Source of variation	d.f.	Variance component	% Variance	Φ statistics	*P*-value
Site	2	0.3598	4.98	0.049	0.1998
Host species within site	3	1.3321	18.46	0.194	0.0117
Within host species	109	5.5254	76.56	–	–

fluctuated among sites, being highest between *H. foetidus*-*P. vulgaris* in LC ($\Phi = 0.377$, $P = 0.017$), intermediate between *H. foetidus*-*N. cuatrecasasii* in FB ($\Phi = 0.151$, $P = 0.056$), and lowest between *H. foetidus*-*N. cuatrecasasii* in RV ($\Phi = 0.017$, $P = 0.19$).

Conspecific host plants

It was possible to test whether locally coexisting, conspecific host plants harbored genetically distinct subpopulations of *M. reukaufii* using the data from *H. foetidus* plants sampled at FB and RV in 2010, for which a sufficient number of individual hosts and yeast isolates were available (40 isolates from eight host plants at each site). In each site, *M. reukaufii* isolates from the seven most frequent genotypes were nonrandomly distributed among *H. foetidus* plants ($P \ll 0.0001$ in both FB and RV sites; separate Fisher's exact probability tests). In addition to such nonrandom distribution of genotypes among conspecific hosts, there was also a highly significant genetic variance component associated with conspecific host plants within sites, as revealed by the hierarchical AMOVA on the pairwise genetic distance matrix between isolates (Table 3). At a given site, therefore, flowers borne by different *H. foetidus* plants tended to harbor significantly different genetic subpopulations of *M. reukaufii*.

Discussion

The study of mechanisms allowing for the persistence of genetic variation in the face of the eroding action of natural selection is particularly relevant to evolutionary genetics theory in the case of asexually reproducing organisms (Williams, 1975; Halkett *et al.*, 2005). The present study has shown that natural populations of the clonal yeast *M. reukaufii* harbor considerable genotypic diversity, as found previously for other asexual organisms, including plants, animals, protists, and other yeasts (e.g. Ellstrand & Roose, 1987; Anderson & Kohn, 1995; Halkett *et al.*, 2005; Herrera *et al.*, 2011; Llewellyn *et al.*, 2011; Rengefors *et al.*, 2012). It has been long known that spatially varying selection can maintain genetic variation (Levene, 1953; Hedrick, 1986), and diversifying selection has been suggested as a major factor in maintaining genetic variation in clonal organisms (Ellstrand & Roose, 1987; Kassen & Rainey, 2004; Herrera *et al.*, 2011; Llewellyn *et al.*, 2011). If the fitness of different clones differs across microsites and/or environmental conditions, then environmental heterogeneity will favor the persistence of different clonal lineages at different microsites, thus contributing to preserve overall genotypic diversity (Burdon, 1980; Ellstrand & Roose, 1987; Kassen & Rainey, 2004). In the case of clonal microorganisms, experiments in artificial laboratory microcosms have often confirmed that complex, heterogeneous environments are more likely to preserve genotypic diversity than simpler, homogeneous ones (Kassen & Rainey, 2004; Replansky & Bell, 2009). Testing the diversifying selection hypothesis in natural environments, however, confronts difficulties arising from identifying which microhabitat patches are relevant from the microorganisms' viewpoint. Specialized nectar yeasts such as *M. reukaufii* are favorable systems in this respect, as the relevant microhabitat patches (flowers) are easily recognizable macroscopically. In addition, nectar chemistry (e.g. pH, sugar concentration, sugar composition) of *M. reukaufii* hosts in our study region varies widely between flowers of the same and different plant species (Herrera & Soriguer, 1983; Herrera *et al.*, 2006, 2012; C. M. Herrera, unpublished data), which provides a suitable ecological background for testing the diversifying selection hypothesis.

By genotyping *M. reukaufii* isolates obtained from flowers of different plant species and individuals of the same species, this study has shown a strong host-mediated component of genetic and genotypic diversity at all scales considered. Despite reduced statistical power due to small number of isolates in some samples (Ryman *et al.*, 2006), our results indicate that *M. reukaufii* genotypes were non-randomly distributed among flowers of different species and, in the case of *Helleborus foetidus*, also among flowers of different individuals coexisting locally. The AMOVA analyses also revealed significant components of genetic variance associated with different host species and even individual plants of the same species. These results are compatible with the diversifying selection hypothesis for the maintenance of local and regional genetic diversity in clonal *M. reukaufii*, although a comprehensive test would also require unraveling the mechanisms that generate such patterns (Hedrick, 1986).

Differential colonization/migration (between flowers) and selection (within flowers) should ultimately account for nonrandom genotype distribution among floral hosts

Table 3. Hierarchical AMOVA on the pairwise matrix of genetic distances between the 80 *Metschnikowia reukaufii* isolates obtained in 2010 from nectar of 16 *Helleborus foetidus* host plants at the FB and RV sites

Source of variation	d.f.	Variance component	% Variance	Φ statistics	*P*-value
Site	1	0.6792	11.18	0.112	0.033
Individual host within site	14	2.1189	34.89	0.393	< 0.0001
Within individual host	64	3.2750	53.93	–	–

of *M. reukaufii* observed at the spatial and temporal scale of this study. In addition to being associated with transient habitat patches (individual flowers), metapopulations of specialized nectar yeasts such as *M. reukaufii* are themselves ephemeral as well, because they collapse when flowers disappear at the end of the blooming season. Until the next flowering period, nectar yeasts remain dormant associated with overwintering floricolous insects, their brood, or their nest provisions (Spencer *et al.*, 1970; Inglis *et al.*, 1993; Brysch-Herzberg, 2004). Flowers are recolonized as they reappear in early spring. As pollinators are the exclusive vectors of nectar yeasts, and flowers of different plant species tend to be visited by different pollinators, nonrandom associations between yeast genotypes and major pollinator types (e.g. bumble bees vs. large solitary bees in the present study; Table 1) should translate into biased colonization/migration and subsequent segregation of genotypes with regard to plant species. The genetic differences between *M. reukaufii* isolates associated with pairs of locally coflowering host species with different pollinators are compatible with this mechanism. It is unlikely, however, that biased colonization/migration of genotypes was the only factor responsible for patterns of genetic diversity found in this study. Genetic differences between *M. reukaufii* isolates from *H. foetidus* plants growing side by side and sharing the same pollinators are difficult to explain exclusively in terms of differential genotype colonization of different host individuals. In addition, when cultured in the laboratory in artificial nectaries containing natural floral nectar from different species and different individuals of the same species, cell multiplication rate of *M. reukaufii* depended on genotype and nectar provenance (plant species and individual; C. M. Herrera, unpublished data). Very fine-scale, intrafloral divergent selection has been documented for *M. gruessii*, another nectar-living yeast (Herrera *et al.*, 2011). We thus tentatively suggest that a combination of intrafloral selection and pollinator-mediated, biased migration/colonization may account for the nonrandom distribution *M. reukaufii* genotypes among host plants in our study area. Further experiments will be needed to validate this hypothesis.

From a practical viewpoint, results of this study highlight the importance of sampling a range of flower hosts to obtain realistic genetic diversity estimates for specialized flower-dwelling yeasts. Our results are also relevant to the more general issue of the relationship between microbial genetic diversity and plant community ecology. Recent studies documenting connections between genetic diversity of populations and functioning of ecological communities have rarely examined complex ecological systems (Hughes *et al.*, 2008; Hersch-Green *et al.*, 2011). In particular, the possible cascading effects involved in plant–animal mutualistic assemblages that revolve around plants provisioning food for animals (pollination, seed dispersal) remain unexplored. Our results suggest that local and regional diversity of flowers and pollinators may have discernible top-down effects on the amount and structuring of genetic diversity of the microorganisms that specialize on the exploitation of such systems. It can be conjectured that the latter's genetic structure and diversity should be contingent on the diversity of pollination systems and floral nectar features represented in local and regional plant communities and that declines in microbial genetic diversity should be expected from the current impoverishment of natural plant–pollinator systems (Kearns *et al.*, 1998).

Conflict of interest

The authors declare no conflict of interests.

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