Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast

C. M. HERRERA, M. I. POZO and P. BAZAGA

Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Américo Vespucio s/n, Isla de La Cartuja, 41092 Sevilla, Spain

Abstract

In addition to genetic differences between individuals as a result of nucleotide sequence variation, epigenetic changes that occur as a result of DNA methylation may also contribute to population niche width by enhancing phenotypic plasticity, although this intriguing possibility remains essentially untested. Using the nectar-living yeast *Metschnikowia reukaufii* as study subject, we examine the hypothesis that changes in genome-wide DNA methylation patterns underlie the ability of this fugitive species to exploit a broad resource range in its heterogeneous and patchy environment. Data on floral nectar characteristics and their use by *M. reukaufii* in the wild were combined with laboratory experiments and methylation-sensitive amplified polymorphism (MSAP) analyses designed to detect epigenetic responses of single genotypes to variations in sugar environment that mimicked those occurring naturally in nectar. *M. reukaufii* exploited a broad range of resources, occurring in nectar of 48% of species and 52% of families surveyed, and its host plants exhibited broad intra- and interspecific variation in sugar-related nectar features. Under experimental conditions, sugar composition, sugar concentration and their interaction significantly influenced the mean probability of MSAP markers experiencing a transition from unmethylated to methylated state. Alterations in methylation status were not random but predictably associated with certain markers. The methylation inhibitor 5-azacytidine (5-AzaC) had strong inhibitory effects on *M. reukaufii* proliferation in sugar-containing media, and a direct relationship existed across sugar $\times$ concentration experimental levels linking inhibitory effect of 5-AzaC and mean per-marker probability of genome-wide methylation. Environmentally induced DNA methylation polymorphisms allowed genotypes to grow successfully in extreme sugar environments, and the broad population niche width of *M. reukaufii* was largely made possible by epigenetic changes enabling genotype plasticity in resource use.

Keywords: DNA methylation, epigenetics, floral nectar, *Metschnikowia reukaufii*, nectar composition, nectar sugars, niche width, phenotypic plasticity

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Introduction

The concept of niche width, which refers to the variety of resources exploited by a population, has persisted for decades in the focus of ecological and evolutionary research because of its conceptual connections with the generation and maintenance of biological diversity (Hutchinson 1959; Levins 1968; Roughgarden 1972; Futuyma & Moreno 1988; Kassen 2002; Bolnick *et al.* 2007; Ricklefs 2010). Over the years, it has been recurrently emphasized that ecological and evolutionary implications of population niche width will depend on the magnitude of its between- and within-genotype components (Van Valen 1965; Roughgarden 1972; Bolnick *et al.* 2007). In this context, a central question of

Correspondence: Carlos M. Herrera, Fax: +34 954 621125;
E-mail: herrera@ebd.csic.es

longstanding interest has been to understand the degree to which populations with broad niches are composed of specialized individuals each exploiting a different resource range (i.e. large between-genotype component) or generalized individuals all possessing similarly broad niches (i.e. large within-genotype component) (Roughgarden 1972; Futuyma & Moreno 1988; Bolnick *et al.* 2007; Scriber 2010). Theoretical models suggest that components of population niche width should be adaptively related to features of the organisms and their environments. It has been predicted, for example, that the between-genotype component should increase following release from interspecific competition ('niche variation hypothesis'; Van Valen 1965; Roughgarden 1972; Bolnick *et al.* 2007). There are also theoretical expectations that broad within-genotype niches achieved through phenotypic plasticity should evolve in regularly colonizing species with a metapopulation structure, and in those exploiting fine-grained habitats where environmental factors vary on spatial scales that are smaller than dispersal distances (Roughgarden 1972; Sultan & Spencer 2002; Baythavong 2011). Under these circumstances, broad population niche widths would mainly reflect the ability of individual genotypes to exploit broad resource ranges because of extensive phenotypic plasticity (Roughgarden 1972).

While the environmental and life-history correlates of phenotypic plasticity are reasonably well understood theoretically (Pigliucci 2001; DeWitt & Scheiner 2004), empirical tests often confront difficulties. Obtaining replicates of experimental units with identical genotypes for directly assessing single-genotype responses to variable environments is often problematic with multicellular organisms that reproduce sexually, such as the majority of animals and plants. This limitation has been traditionally circumvented by adoption of quantitative genetic models, which use a statistical approach to evaluate within-genotype components of population responses to variable environments (Scheiner 1993). This 'black box approach', however, is unsuitable to deal with the mechanistic basis of phenotypic plasticity (Pigliucci 1996). Recently, novel molecular tools have opened unprecedented opportunities for understanding the genetic mechanisms allowing individual genotypes to successfully cope with variable environments (Pigliucci 2001; Aubin-Horth & Renn 2009). There have been also suggestions that in addition to genetic factors related to nucleotide sequence variation, epigenetic variations as a result of changes in DNA methylation may contribute to phenotypic plasticity in plants and fungi (Bossdorf *et al.* 2008, 2010; Slepecky & Starmer 2009), but this intriguing possibility remains essentially unexplored to date (Richards *et al.* 2010; Richards 2011). Properly testing the epigenetic basis of niche

width and phenotypic plasticity, however, is not exempt from difficulties as it requires teasing apart epigenetic and genetic effects, a challenging task in natural populations of sexually reproducing organisms (Herrera & Bazaga 2011; Bossdorf & Zhang 2011). In this respect, unicellular fungi are particularly favourable study systems for investigating the epigenetic basis of plasticity, as they allow for virtually unlimited cloning of genotypes for multiple testing in contrasting environments (Slepecky & Starmer 2009).

In this study, we test theoretical expectations on genotype plasticity and explore its underlying molecular mechanisms in the nectar-living yeast *Metschnikowia reukaufii* (Ascomycota, Metschnikowiaceae). Our approach combines field data on resource use with laboratory experiments designed to detect epigenetic responses by genotypes to changes in the sugar environment mimicking natural variation in floral nectar. The following two hypotheses will be specifically addressed: (i) fugitive species, '…forever on the move, always becoming extinct in one locality… and always surviving by reestablishing themselves in some other locality as a new niche opens' (Hutchinson 1951, p. 575), that exploit patchy, extremely fine-grained environments and have a strong metapopulation structure, should simultaneously evolve broad ecological niches and considerable plasticity in resource use and (ii) changes in patterns of genome-wide DNA methylation in response to variable environments should underlie the ability of individual genotypes to exploit broad resource ranges (i.e. plasticity).

Several ecological and life-history features render *M. reukaufii* an ideal study subject for testing these hypotheses. The species is known from several continents, and it has been always associated with flowers or floral nectar (Lachance *et al.* 2001; Brysch-Herzberg 2004; Pozo *et al.* 2011). Colonizing inocula are brought to flowers by foraging pollinators (Brysch-Herzberg 2004; Herrera *et al.* 2010). Cells proliferate asexually in floral nectar, often reaching densities $>10^5$ cells/mm^3 (Herrera & Pozo 2010). As found for its congeneric, the also nectar-living *Metschnikowia gruessii* (Herrera *et al.* 2011), sexual reproduction does not occur naturally in flowers, where populations are exclusively clonal (C. M. Herrera, M. I. Pozo & C. de Vega, unpublished). From the viewpoint of a nectar specialist like *M. reukaufii*, the environment consists of discrete, tiny patches of suitable habitat each corresponding to a single flower. As flowers are ephemeral structures, regional *M. reukaufii* populations will inevitably function as a metapopulation with high patch turnover rates and intense colonization-extinction dynamics. Regional persistence will require continuous reiteration of colonization-multiplication-dispersal cycles as new host flowers appear and disappear from the

habitat. Colonization and dispersal stages will depend on patterns and frequency of pollinator movements between flowers, while successful multiplication in these short-lived patches will require an ability to quickly proliferate following pollinator-mediated transfer to nectar droplets. As nectar chemistry varies between flowers of the same and different species (e.g. Herrera *et al.* 2006; Nicolson & Thornburg 2007), proliferation in newly colonized nectar droplets will depend critically on the yeast's ability to cope with a broad range of nectar environments. As synthesized by Roughgarden (1972, p. 711), 'since an asexual population cannot generate variability through recombination, it must carry variability with it to the new environment.' Our hypothesis, that populations of *M. reukaufii* should have broad ecological niches and individual genotypes should exhibit a high degree of phenotypic plasticity, is motivated by the preceding natural history considerations in combination with predictions of niche width theory for metapopulations of colonizing species in fine-grained environments (Roughgarden 1972; Sultan & Spencer 2002; Baythavong 2011).

Materials and methods

Field methods

Field sampling and observations reported in this study were done during March-September 2007–2011 in several localities within a relatively small (∼25 km^2) area in the Sierra de Cazorla region, Jaén province, southeastern Spain (see the studies by Herrera *et al.* 2009, 2011; Pozo *et al.* 2011; for sampling sites). To assess host plant use by *Metschnikowia reukaufii*, a total of 2359 nectar samples from 94 plant species in 31 families were collected from flowers which had been exposed to natural visitation by pollinators and examined microscopically following methods described in Herrera *et al.* (2009) and Pozo *et al.* (2011). For the purposes of this study, we will consider only presence/absence of *M. reukaufii* in nectar samples, irrespective of cell density. Given the large number of nectar samples examined, it was prohibitive to apply to all samples the standard culturing, isolation and DNA sequencing methods required for rigorous yeast species identification. Instead, we relied on our familiarity with the distinctive morphological features of *M. reukaufii* cells and cell groups to ascertain its presence in nectar samples. The accuracy of morphology-based species determination was cross-checked by DNA sequencing (as described by Pozo *et al.* 2011) yeast isolates from a subset of $N = 140$ nectar samples from 20 plant species. Samples were streaked onto yeast malt + chloramphenicol agar plates, isolates were obtained from the resulting colonies, and the D1/D2 domain of the 26S

ribosomal RNA gene was partially sequenced following methods proposed by Kurtzman & Robnett (1998) and Lachance *et al.* (1999). Results were compared with the corresponding sequence for *M. reukaufii* type strain (CBS5834) in GenBank (accession number U44825.1). Molecular identifications always matched morphological identifications.

The nectar sugar environment naturally faced by *M. reukaufii* in the study area, and extent of pollinator sharing by simultaneously flowering hosts, was studied in a focal subset of 12 host species flowering during March-June. Natural variation in sugar concentration and sugar composition of floral nectar was determined for six and four of these focal species, respectively. For each species, nectar samples were collected on separate dates from flowers of different plants exposed to natural pollinator visitation and analysed following methods described in the next section (sample sizes are given in the Results). To evaluate the degree of pollinator sharing and thus indirectly the likelihood of between-host dispersal and colonization by pollinator-borne *M. reukaufii*, pollinator watches were undertaken on flowering individuals of the 12 focal species following methods proposed by Herrera (2005). Proportions of total flower visits contributed by different pollinators to each host plant were estimated from these data and used to evaluate similarity between simultaneously flowering species in pollinator composition. Three groups of species were distinguished according to flowering time (March–April, May and June). Species in each group coexisted locally, so that pollinator similarity could be interpreted as an indirect measure of the likelihood of yeast dispersal between host plants, and field observations revealed that individual pollinators often visited flowers of different species consecutively.

Laboratory methods: nectar features

Floral nectar of species included in the study of variation in sugar characteristics was extracted using calibrated microcapillaries. Samples were stored for analyses of sugar composition (absorbed into chromatography paper wicks or frozen in microcapillaries) or used immediately for determining total sugar concentration to the nearest 0.5% with a temperature-compensated, handheld refractometer adapted for small volumes (Eclipse Professional model, Bellingham and Stanley Ltd., Tunbridge Wells, Kent, UK). Nectar sugar concentration measurements were expressed as per cent sucrose equivalents on a weight-to-weight basis. Nectar sugar composition of stored samples was analysed with high-performance liquid chromatography (HPLC; see the study by Herrera *et al.* 2006 for details). The

proportions in each analysed sample of the three main sugars (glucose, fructose and sucrose) were estimated by integrating the areas under chromatogram peaks. Trace amounts of other sugars appearing only rarely will not be considered here.

Laboratory methods: yeast cultures

Experiments were conducted to test the hypotheses that (i) genome-wide DNA methylation patterns of individual *M. reukaufii* genotypes varied in response to changes in sugar composition and concentration and (ii) DNA methylation changes experienced by individual genotypes in response to variable environmental conditions were instrumental in allowing them to exploit a broad range of sugar environments. Five *M. reukaufii* strains obtained in the study area from floral nectar of five different host species were used in the experiments. All strains were genotypically distinct, as confirmed by DNA fingerprinting with amplified fragment length polymorphism (AFLP) markers following the methods of Herrera *et al.* (2011).

Strains were replica-plated on media differing in sugar concentration and sugar composition, and cultured at 25 °C. A broad experimental 'sugar environment' was generated by adding different sugars at variable concentrations to yeast extract agar (YE hereafter; 1.0% yeast extract, 2.0% agar) according to a two-way factorial design. Combinations of sugar (three levels: glucose, fructose and sucrose) and concentration (four levels: 20%, 30%, 40% and 50%) factors led to a 12-level sugar environment mimicking that faced naturally by *M. reukaufii* in the floral nectars of our study area. Strains were also cultured in yeast malt agar (YM hereafter; 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1.0% glucose, 2.0% agar), which served as a low-sugar control environment. Whenever colony growth provided sufficient material, repeated DNA samples were obtained for every strain in each experimental growth media (including YM) on days 2, 7, 12 and 17 after plating. A total of 219 usable DNA samples (214 experimental + five YM controls at day 2) were eventually obtained, all of which were fingerprinted using a simplified version of the methylation-sensitive amplified fragment polymorphism (MSAP) technique described later.

In a related experiment, the preceding two-way factorial design was expanded to a three-way one. The third factor involved the nucleoside analogue 5-azacytidine (5-AzaC hereafter), an inhibitor of DNA methylation in eukaryotes including fungi. For each sugar composition × sugar concentration level, the five experimental strains were cultured in media with and without 300 μM 5-AzaC, so that a three-way, 24-level (three sugar types × four concentrations × two 5-AzaC levels) factorial design was eventually obtained. Strains were also cultured in YM agar with and without 5-AzaC for reference. Colony growth was scored on an ordinal scale from 0 (no growth) to 4 (maximum growth, thickest and broadest colony) on days 2, 7, 12 and 17 after plating. All scoring was done by the same person (MIP). Beyond a certain concentration threshold, 5-AzaC leads to cytotoxicity and cell cycle arrest in some yeasts (Taylor *et al.* 1996). As such strong effects might distort or obscure the results of the experiment, a 25–1000 μM range of 5-AzaC concentrations was first screened for their effect on the growth of dilute yeast suspensions of the five strains used in the experiments swabbed individually in yeast nitrogen base agar plates (YNB, Difco, 0.67% of yeast nitrogen base agar, 1% glucose, 1.5% agar) using a disc-diffusion assay. The 300-μM experimental concentration chosen had a weak but still measurable effects on cell multiplication.

Laboratory methods: DNA methylation

Cytosine methylation of fungal DNA mostly occurs in CCGG sequences (Magill & Magill 1989; Freedman & Pukkila 1993); hence, restriction enzymes that recognize this sequence and are affected by the methylation status of its cytosine residues can be used to study genome-wide profiles of DNA methylation. The ordinary MSAP technique is a modification of the AFLP method that identifies methylation-susceptible anonymous 5′-CCGG sequences and assess their methylation status by comparing band patterns obtained with paired primer combinations containing either of the isoschizomers *Hpa*II or *Msp*I as frequent cutter (see e.g. Reyna-López *et al.* 1997; Cervera *et al.* 2002; Herrera & Bazaga 2010, 2011; for examples). As we were interested in detecting DNA methylation events experienced by individual genotypes (i.e. within-genotype methylation polymorphisms) rather than on methylation differences between genotypes, a simplified MSAP method was adopted in which only primer combinations with the methylation-sensitive *Hpa*II were used. *Hpa*II cleaves CCGG sequences but is inactive when either or both cytosines are fully methylated, and cleaving may be impaired or blocked when one or both of the cytosines are hemi-methylated (McClelland *et al.* 1994; Roberts *et al.* 2007). In the absence of genetic (sequence) variation among DNA samples, therefore, polymorphism of MSAP markers will reflect sample heterogeneity in the methylation status of the associated CCGG site, with 0 to 1 and 1 to 0 changes denoting, respectively, demethylation and methylation events (see the study by Verhoeven *et al.* 2010 for an application of this simplified MSAP method). We will be exclusively concerned here with methylation events.

Four *Hpa*II/*Mse*I primer combinations, each with two (*Hpa*II) or three (*Mse*I) selective nucleotides, were used for fingerprinting experimental samples: *Hpa*II + TC/*Mse*I + CTT, *Hpa*II + TT/*Mse*I + CAC, *Hpa*II + TA/*Mse*I + CAT and *Hpa*II + TG/*Mse*I + CCC. Analyses were performed essentially as described originally by Vos *et al.* (1995), with modifications involving the use of fluorescent dye-labelled selective primers following Applied Biosystems (2005). Fragments were separated and detected using an ABI PRISM 3130xl DNA sequencer, and the presence/absence of each marker in each sample was scored manually by visualizing electrophoregrams 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). The four *Hpa*II/*Mse*I primer combinations produced a total of 325 MSAP markers that could be reliably scored. Only the 250 markers that were present in 2–98% of the samples were retained for analyses of genome-wide methylation changes.

Data analysis

Prior to the analyses, MSAP marker scores for experimental samples were transformed by comparing with the corresponding values (i.e. same strain and marker) for the samples grown in the YM reference medium. Deviations of MSAP marker scores from YM to experimental samples involving a change from 1 to 0 would denote a methylation event of the marker involved. A new sample ($N = 214$) × marker ($N = 250$) score matrix (transformed MSAP matrix, tr-MSAP hereafter) was obtained where each element denoted whether the experimental sample involved had experienced (score = 1) or not (score = 0) a methylation event in the corresponding marker. Non-zero elements of the tr-MSAP matrix thus denoted instances of within-genotype methylation polymorphisms.

The effect of the sugar environment on genome-wide methylation patterns was tested by modelling the per-marker probability of occurrence of within-genotype methylation polymorphism as a binomial process using a generalized linear mixed model framework (Jiang 2007). Our analytical approach (see also the study by Verhoeven *et al.* 2010) is particularly well suited to test for significance of environmental effects on within-genotype methylation changes because of the property of linear models of taking into account uncertainty in the dependent variable arising from unobservable random errors (Jiang 2007), such as those arising during genotyping. A generalized linear mixed model was fitted to data in the tr-MSAP matrix, with occurrence of a methylation event as the dependent variable and sugar type, sugar concentration and their interaction as independent variables. Epigenotypes of the same strain on different sampling dates, and methylation events of the same marker, are likely to be correlated. To statistically account for these potential sources of non-independence, the model included strain identity, day after plating and marker as random effects. Strain identity was treated as a random effect because we were not interested here in differences between genotypes in methylation response to the sugar environment. We did assess, however, the possible heterogeneity of genotypes in experimental responses by comparing with zero the component of variance associated with strains by means of an approximate Z-score asymptotic test (Littell *et al.* 1996). Computations were performed using SAS procedure GLIMMIX, with binomial distribution for errors, logits as link function, residual pseudo-likelihood estimation, and the default containment method for computing denominator degrees of freedom (SAS Institute 2006). Model-adjusted least squares means and standard errors of response variables for different treatment levels were obtained with the LSMEANS statement and the ILINK option. The significance of 'simple main effects', that is, the effects of a given factor at different treatment levels of the other factor (Pedhazur 1982), was tested with the SLICE option.

To determine whether genome-wide changes in DNA methylation occurred randomly or predominantly affected some specific markers, separate generalized linear mixed models were fit for each MSAP marker, with occurrence of a methylation event as the dependent variable and sugar type, sugar concentration and their interaction as the independent variables. Strain and days after plating were included as random effects. To account for increased probability of Type I errors (false positives) due to the multiplicity of statistical tests, the *q*-value method for estimation of false discovery rates was applied to the set of *P*-values obtained from these models (Storey & Tibshirani 2003). Using the QVALUE package (Storey & Tibshirani 2003), we found the largest *q*-value leading to an expectation of less than one falsely significant effect [i.e. *q*-value × (number of effects accepted as significant) < 1].

Generalized linear mixed models and procedure GLIMMIX were also used for testing significance of effects of sugar environment (composition and concentration) and 5-AzaC methylation inhibitor on *M. reukaufii* growth. Sugar composition, sugar concentration and 5-AzaC were included as main fixed effects, and strains and days after plating as random effects. Owing to the ordinal nature of the index used to score growth, multinomial distribution of errors and cumulative logits link function were used (Agresti 2007).

Results

Host plant use

Metschnikowia reukaufii exploited a remarkably broad range of floral nectar resources in the study area. Over March–September, the species was found in nectar of 45 plant species from 16 families (Table S1), which represented 48% and 52% of species and families surveyed, respectively. Frequency of occurrence of *M. reukaufii* in nectar varied widely among hosts, ranging between 5–100% (mean ± SE = 44.4 ± 4.2%, N = 45 species; Table S1). Throughout the blooming season, locally coexisting hosts that flowered simultaneously often shared pollinators to a considerable extent, as illustrated by high pairwise similarities in pollinator composition in the 12 host plants investigated (Fig. S1) and by the fact that these often shared their most frequent pollinators (Table S2).

Nectar variation

Host plants of *M. reukaufii* exhibited broad intra- and interspecific variation in sugar-related features of floral nectar, as exemplified by the six species sampled. These species differed widely in central tendency of the distributions of sugar concentration (Fig. 1), with means (±SE) ranging between 16.2 ± 0.2 (*Digitalis obscura*) and 47.8 ± 18.9 (*Primula vulgaris*) per cent sucrose equiva-
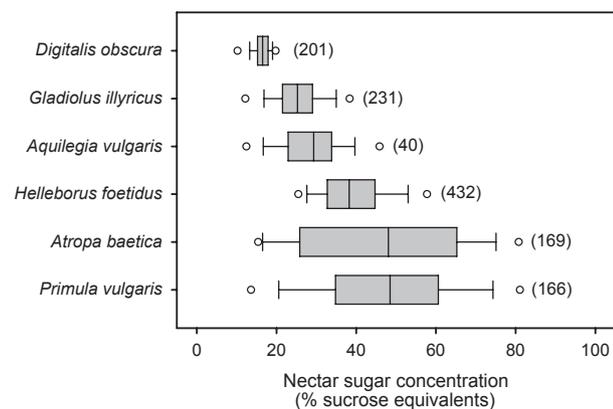

Fig. 1 Intra- and interspecific variation in nectar sugar concentration (per cent sucrose equivalents on a weight-to-weight basis) in six representative host plants of *Metschnikowia reukaufii* in the study area. In each boxplot, the lower and upper boundaries of the box indicate the 25th and 75th percentiles, the line within the box marks the median, whiskers indicate the 10th and 90th percentiles, and circles denote the 5 and 95 percentiles of distributions. Figures in parentheses represent the total number of single nectary (*Helleborus foetidus* and *Aquilegia vulgaris*) or single-flower (rest of species) nectar samples examined.

lents, an approximately threefold variation. Intraspecific variation in sugar concentration was also extensive in some species. In two of these (*Atropa baetica*, *Primula vulgaris*), the range of intraspecific variation encompassed nearly the full possible spectrum (20–80% sucrose equivalents) and was comparable to that for all species combined (Fig. 1). Partitioning total variance in sugar concentration of the combined sample for all species (N = 1239 measurements) into its within- and among-species components revealed that 50.7% was accounted for by variation between flowers of the same species.

Nectar sugar composition of *M. reukaufii* hosts was also highly variable within and among species as revealed by HPLC analyses (Fig. 2). On average, sucrose was the dominant sugar in three of the species studied, and fructose in the other, although all species exhibited extensive variation among samples in relative proportions of individual sugars. In *Aquilegia vulgaris* and *Helleborus foetidus*, for instance, sugar composition of nectar samples fell along a broad gradient running from pure sucrose to pure fructose (Fig. 2).

Sugar environment and DNA methylation

The probability of MSAP markers exhibiting within-genotype methylation events in experimental samples of *M. reukaufii* relative to the corresponding YM reference sample was modelled by fitting a generalized linear mixed model with within-genotype methylation as dependent variable, and sugar composition and concentration as fixed main effects. The model fitted the data well, as shown by a ratio of generalized chi-square statistic to degrees of freedom near unity (0.67). The variance component associated with differences between strains did not differ significantly from zero (Z-score = 1.40, Table 1), which denotes homogeneity of genotypes in DNA methylation responses.

After statistically accounting for the influence of random effects, the probability of a MSAP marker experiencing a within-genotype change from the unmethylated to the methylated state was significantly related to sugar composition, sugar concentration, and their interaction (Table 1). Mean per-marker DNA methylation probability was highest when *M. reukaufii* was grown with fructose, lowest when grown with sucrose, and intermediate when grown with glucose (Fig. 3). Methylation probability was also significantly related to sugar concentration, with a minimum occurring at 30% and a distinct maximum at 50% (Fig. 3). Significance of the sugar × concentration interaction was mainly the consequence of the steeper increase in methylation probability with increasing concentration when yeasts were grown with fructose (Fig. 3).
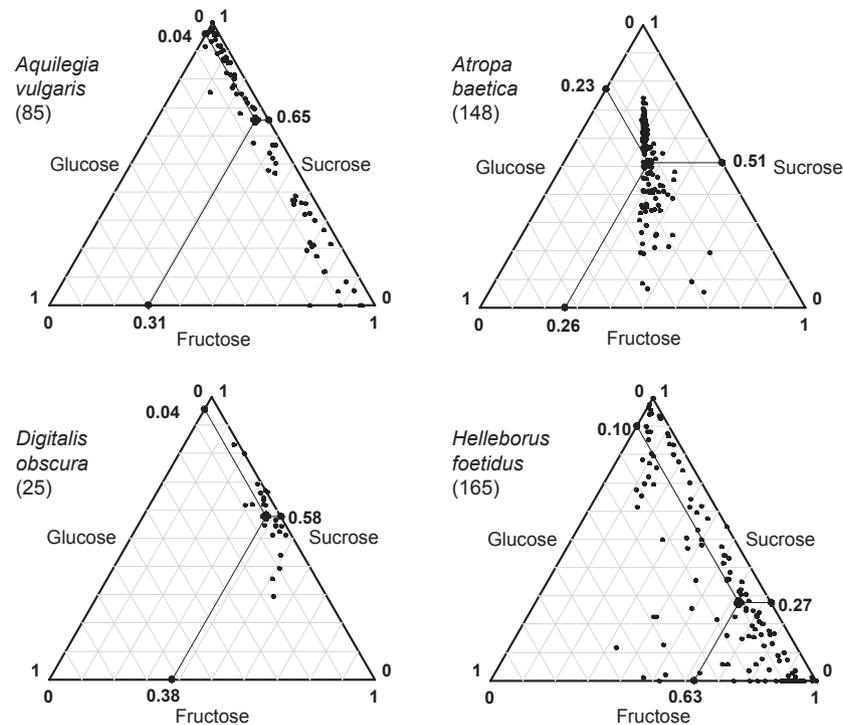
Fig. 2 Ternary diagrams showing the distribution of nectar samples of four host plants of *Metschnikowia reukaufii* over the plane defined by the proportions of glucose, fructose and sucrose. Each point depicts the proportional sugar composition of nectar from a single nectary (*Helleborus foetidus*, *Aquilegia vulgaris*) or flower (*Atropa baetica*, *Digitalis obscura*). Averages are also shown, with mean proportion of each sugar indicated on the corresponding axis. Sample sizes in parentheses beside species names. Graphs for *H. foetidus* and *A. vulgaris* include data from the study by Herrera *et al.* (2008).

Table 1 Summary of results of the generalized linear mixed model fitted to experimental *Metschnikowia reukaufii* data, with within-genotype methylation polymorphism as the dependent variable, sugar composition (fructose, glucose, sucrose) and concentration (20%, 30%, 40%, 50%) as fixed main effects, and methylation-sensitive amplified polymorphism (MSAP) marker, strain and days after plating as random effects

Effects	F	d.f.	P
Fixed:			
Sugar (S)	56.40	2,53232	<0.0001
Concentration (C)	39.07	3,53232	<0.0001
S × C	20.15	6,53232	<0.0001
	Variance	Standard error	Z-score
Random:			
MSAP marker	4.4856	0.4929	9.10
Strain	0.1627	0.1160	1.40
Days after plating	0.1058	0.08738	1.21

It was possible to fit separate marker-by-marker generalized linear mixed models testing for the effects of sugar, concentration and their interaction on methylation probability for 120 different MSAP markers. Models failed to converge for the rest of markers, presumably because of extreme imbalance of binomial response levels, and these results were discarded. Among successful model fits, and after controlling for increased Type I error, a total of eight markers (6.7%) were found that exhibited significant effects of sugar composition, concentration and/or their interaction on their probability of experiencing methylation (Table 2). Individual markers differed widely in their methylation responses to variations in the sugar environment. For example, two markers were responsive simultaneously to concentration, composition and their interaction, while three markers were responsive to changes in concentration alone (Table 2).

Sugar and 5-AzaC effects on growth

Growth of *M. reukaufii* was significantly affected by sugar composition and concentration (Table 3). Genotypes were similar in their experimental responses, as denoted by the nonsignificant variance component due to strains (Z-score = 0.50, Table 3). Sucrose was the most favourable sugar for growth, fructose the worst one and glucose was intermediate (Fig. S2). There was
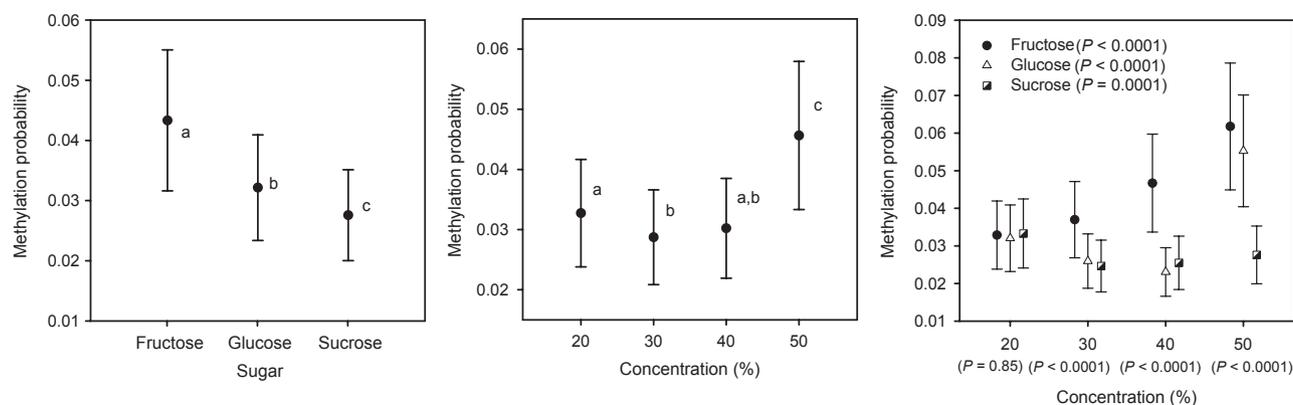
Fig. 3 Model-adjusted, least squares means (±1 SE) for methylation-sensitive amplified polymorphism marker methylation probability in experimental samples of *Metschnikowia reukaufii* for different levels of main factors (sugar, left graph; concentration, middle graph) and their interaction (right graph). Means in the same graph sharing a letter are not statistically different (adjusted $P > 0.05$). In the interaction graph, P-values in parentheses beside sugar and concentration levels refer to the significance of simple main effects.

Table 2 Methylation-sensitive amplified polymorphism (MSAP) markers that experienced statistically significant methylation polymorphisms following experimental exposure of *Metschnikowia reukaufii* genotypes to growing environments differing in sugar concentration, composition and sugar × concentration combinations

Marker†	Effect (P)		
	Concentration (C)	Sugar (S)	C × S
*Hpa*TC_*Mse*CTT_216	0.0062	0.0095	0.0076
*Hpa*TC_*Mse*CTT_217	0.0075		
*Hpa*TC_*Mse*CTT_310	0.0057	0.0065	0.0039
*Hpa*TC_*Mse*CTT_324	0.0034		
*Hpa*TT_*Mse*CAC_192	0.0098		0.0020
*Hpa*TT_*Mse*CAC_389		0.0046	0.0033
*Hpa*TA_*Mse*CAT_157		0.0052	0.0093
*Hpa*TG_*Mse*CCC_496	0.0013		

†Identified by primer combination and fragment size (base pairs).

Table 3 Results of fitting a generalized linear mixed model to experimental data testing for the effects on *Metschnikowia reukaufii* growth of sugar composition (fructose, glucose or sucrose), concentration (20%, 30%, 40% or 50%) and the DNA methylation inhibitor 5-azacytidine (with and without) as fixed main effects, and strain and days after plating as random effects

Effects	F	d.f.	P
Fixed:			
Sugar (S)	92.68	2,458	<0.0001
Concentration (C)	83.50	3,458	<0.0001
5-Azacytidine (A)	107.84	1,458	<0.0001
A × C	8.47	3,458	<0.0001
A × S	5.24	2,458	0.0056
	Variance	Standard error	Z-score
Random:			
Strain	0.03294	0.06529	0.50
Days after plating	7.4616	6.1923	1.20

also a steady decline in growth as sugar concentration increased from 20% through 50%, which took place irrespective of sugar composition (Fig. S2). At 20% sugar concentration, the growth was barely lower, albeit significantly so ($F = 5.05$, d.f. = 1,505, $P = 0.025$), than in YM reference cultures (Fig. S2).

The methylation inhibitor 5-AzaC had no measurable effect on growth when the yeasts were cultured in YM medium ($F = 0.00$, d.f. = 1,31, $P = 1.00$), but it did have a strong, highly significant inhibitory effect in the experimental, sugar-containing media ($F = 28.71$, d.f. = 1,468, $P < 0.0001$; all sugar and concentration treatment levels combined). The magnitude of the effect of 5-AzaC on growth, however, was highly variable,

depending significantly on both sugar composition and concentration, as revealed by the significance of 5-AzaC × sugar and 5-AzaC × concentration interaction effects (Table 3). In relative terms, the deleterious effect of 5-AzaC increased steadily as sugar concentration rose from 20% through 50%. At the lowest sugar concentration assayed (20%), 5-AzaC had no significant effect on growth ($F = 2.21$, d.f. = 1,458, $P = 0.14$). The deleterious effect was strongest for yeasts growing with fructose and glucose, particularly in the 40–50% concentration range, where 5-AzaC completely or nearly completely inhibited growth (Fig. 4).

There was a significant relationship across sugar composition × concentration experimental levels
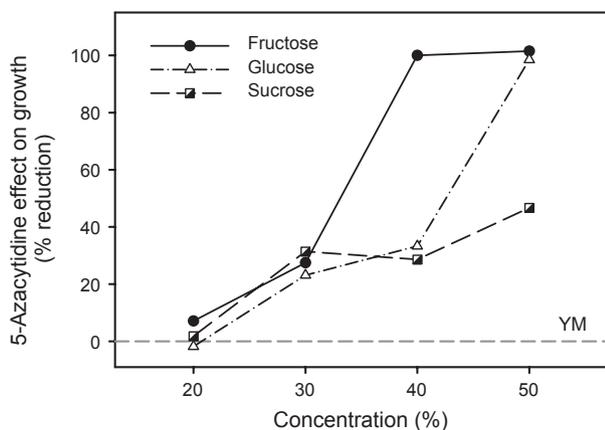
Fig. 4 Relative growth reduction induced by the DNA methylation inhibitor 5-azacytidine (5-AzaC) on *Metschnikowia reukaufii* cultures differing in sugar composition and concentration. Relative growth reduction for a given sugar type × concentration level was computed as the difference between growth indices in media with and without 5-AzaC expressed as a percentage of the latter. Dashed horizontal line denotes baseline effect for yeasts grown in yeast malt reference media. See Table 3 for significance tests of the sugar × 5-AzaC and concentration × 5-AzaC interaction effects on growth, and Fig. S2 for additional information on the dependence of growth on sugar type, sugar concentration and 5-AzaC.
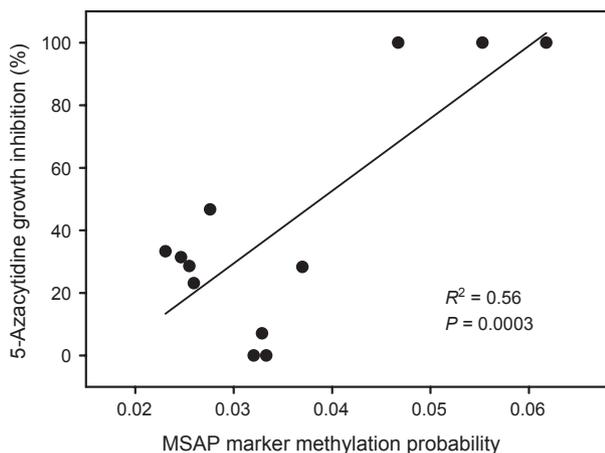

Fig. 5 Relationship across experimental treatment levels between proportional reduction in *Metschnikowia reukaufii* growth induced by the DNA methylation inhibitor 5-azacytidine and mean per-marker DNA methylation probability estimated from methylation-sensitive amplified polymorphism (MSAP) analyses. Each symbol corresponds to a different sugar × concentration experimental combination. The robust regression fitted to the data, and its R^2 and P values are also shown.

between relative inhibitory effect of 5-AzaC on growth at a given level and the corresponding mean genome-wide probability of DNA methylation estimated from MSAP analyses (Fig. 5). Experimental sugar composition × concentration combinations that led to the highest probabilities of MSAP marker methylation were precisely those for which the deleterious effects of 5-AzaC on growth were proportionally strongest.

Discussion

Niche width of Metschnikowia reukaufii

Metschnikowia reukaufii was found in floral nectar of ~50% of the 94 insect-pollinated species sampled for this study. This proportion is similar to that reported for an assortment of nectar-producing plants in a botanical garden in central Germany, where *M. reukaufii* occurred in 48% of samples from 143 host plants (Brysch-Herzberg 2004). With a regional flora comprising ~2100 species of angiosperms (Benavente Navarro 1996), ~78% of which are expected to be insect-pollinated (Ollerton *et al.* 2011) and produce floral nectar, we estimate that in our study region *M. reukaufii* could exploit the floral nectar of ~800 different hosts. Comparable host utilization data are not available for other species of fungal microbes, but the preceding figure stands out by far when compared to host plant diversity of phytophagous animals (e.g. Scriber 2010) and denotes an extraordinary breadth of resource use by this nectarivorous yeast.

A large number of studies have shown that chemical features of floral nectar, including sugar composition and concentration, vary considerably within and between species in natural communities (Baker *et al.* 1998; Petanidou 2005; Chalcoff *et al.* 2006; Herrera *et al.* 2006; Nicolson & Thornburg 2007; among many others). Such variation reflects intrinsic, species-specific differences in nectar characteristics along with the influence of abiotic and biotic environmental factors (Corbet *et al.* 1979; Villarreal & Freeman 1990; Baude *et al.* 2011), including the metabolic activity of nectar-dwelling yeasts (Herrera *et al.* 2008; de Vega *et al.* 2009). Results presented here for nectar sugar concentration and composition confirm this variation for our study region (see also the studies by Herrera & Soriguer 1983; Herrera *et al.* 2006; Canto *et al.* 2007) and suggest that *M. reukaufii* hosts are representative of the regional flora regarding levels of intra- and interspecific variation in sugar-related nectar features (see also the study by Brysch-Herzberg 2004). Even though our study of pollinator composition involved only a modest number of host plants, results suggest that pollinators may transport *M. reukaufii* inocula between flowers of the same or different species and that colonizing propagules will have to cope with a patchy environment where sugar-related features of nectar can be extremely variable from one flower to the next because of both intra- and interspecific differences.

Under laboratory conditions, most species of yeasts respond to variations in the sugar environment comparable or smaller than those found here for the floral nectar environment of *M. reukaufii* in the wild. For example, when glucose and fructose are simultaneously available as carbon sources, some species metabolize preferentially glucose and others fructose (Yu *et al.* 2006; Tronchoni *et al.* 2009). Increasing sugar concentration generally depresses yeast multiplication, and tolerance to high sugar concentrations (e.g. >20%) is a specialized physiological attribute possessed by few species (Tokuoka 1993; Membré *et al.* 1999; Barnett *et al.* 2000; Grant 2004). From the perspective of *M. reukaufii*, extensive variation in sugar composition and concentration in the nectar of its host flowers will bring about considerable between-patch heterogeneity in microhabitat quality, a hypothesis supported by results of the laboratory experiments reported here. Everything else being equal, fructose- or glucose-dominated nectar droplets with concentrations >30% are expected to be inferior microhabitat patches in comparison with, for instance, sucrose-dominated ones with concentrations <40% (Fig. S2). Despite these differences in relative value of microhabitat patches, however, the point we wish to emphasize here is that the high degree of phenotypic plasticity of individual genotypes will enable wild populations of *M. reukaufii* to proliferate successfully in nectars spanning virtually the entire range of natural variation depicted in Figs 1 and 2. In flowers of *Helleborus foetidus*, for example, *M. reukaufii* populations with densities >10^3 cells/mm^3 are commonly found in nectars with concentrations ranging between 15% and 59% sucrose equivalents and proportional sugar compositions ranging between 0–13% for glucose, 26–100% for fructose and 0–70% for sucrose (Herrera *et al.* 2008; C. M. Herrera, unpublished). In addition to corroborating the wide resource use by *M. reukaufii* populations, these field observations confirm that ranges of sugar composition and concentration assayed in our experimental design were comparable to those under which *M. reukaufii* proliferate in the field. Results of laboratory experiments can thus be safely extrapolated to field conditions, particularly the finding that changes in DNA methylation experienced by individual genotypes are causally involved in the ability of *M. reukaufii* to exploit a broad range of nectar environments, as discussed later.

DNA methylation and its consequences

Genome-wide changes in DNA methylation in response to environmental factors have been increasingly shown in recent years for animals (Waterland & Jirtle 2003; Crews *et al.* 2007; Pilsner *et al.* 2010) and plants (Chin-nusamy & Zhu 2009; Peng & Zhang 2009; Verhoeven *et al.* 2010). Similar information is still scarce for fungi, although it is now well established that both ascomycetous and basidiomycetous fungi methylate significant portions of their genomes (Magill & Magill 1989; Binz *et al.* 1998; Selker *et al.* 2003). Furthermore, a number of studies have found correlations between amount and patterns of genome-wide DNA methylation and developmental transitions in fungi (e.g. between unicellular and mycelial stages; Russell *et al.* 1987; Reyna-López *et al.* 1997; Reyna-López & Ruiz-Herrera 2004), which possibly reflect adaptive epigenetic responses to environmental changes (Slepecky & Starmer 2009). We have shown here that genome-wide DNA methylation profiles of individual *M. reukaufii* genotypes changed predictably following exposure to sugar-containing media that mimicked natural variation in nectar sugar composition and concentration. Sugar- and concentration-induced methylation changes involved alterations in both mean per-marker methylation probability and methylation status of individual markers. Rather than being randomly distributed across the genome, alterations in methylation status were predictably targeted to specific zones linked to certain markers, a finding consistent with suggestions that methylated CCGG sequences are not randomly dispersed but rather occur in clusters scattered throughout the fungal genome (Magill & Magill 1989).

Phenotypic and functional consequences of DNA methylation have been traditionally explored through experimental manipulations involving the methylation inhibitor 5-AzaC, and this was also the approach adopted here. In plants, 5-AzaC treatments have repeatedly shown that DNA methylation may be causally linked to, among other, the size, developmental pattern, sexual expression, phenology or floral morphology of individuals (e.g. Sano *et al.* 1990; Janoušek *et al.* 1996; Fieldes & Amyot 1999; Marfil *et al.* 2009; Bossdorf *et al.* 2010). A handful of studies applying the same technique have also revealed that DNA methylation may underlie biochemical, developmental and sex-related phenotypic traits in fungi (Tamame *et al.* 1983a,b; Taylor *et al.* 1996; Filippovich *et al.* 2004; Wang *et al.* 2010). In addition to confirming these earlier investigations, our results for *M. reukaufii* provide also novel insights on the ecological role and adaptive value of inducible DNA methylation in fungi, which can play the important role of enabling individual genotypes to exploit broader resource ranges across patchy, heterogeneous environments.

Exposure to 5-AzaC significantly depressed the growth of *M. reukaufii* in sugar-containing media but had no detectable effect when yeasts were grown in YM reference medium. This finding provides compelling

evidence that the induction of within-genotype polymorphisms in DNA methylation played an important role in enabling *M. reukaufii* to exploit its sugar-dominated, floral nectar environment. In addition, and more importantly, the magnitude of the inhibitory effect of 5-AzaC (i.e. the importance of DNA methylation for yeast multiplication) was strongly context dependent, as it depended simultaneously on the composition and concentration of sugars in the yeast's environment. While the effect of 5-AzaC was not significant at the lowest sugar concentration assayed (20%) irrespective of sugar composition, it increased steadily with increasing sugar concentration, reaching a maximum when yeasts were grown in 40–50% sugar with fructose or glucose as carbon sources (i.e. the role of DNA methylation in cell multiplication was greatest in these conditions). Interestingly, a direct relationship existed across sugar composition × concentration experimental levels between the inhibitory effect of 5-AzaC on growth and the corresponding mean per-marker probability of DNA methylation as detected with the MSAP method: the greater the changes in genome-wide DNA methylation profile, the stronger the impact of 5-AzaC on growth. Taken together, we interpret our experimental results as suggesting that (i) the functional role played by DNA methylation became progressively more important as environmental conditions became harsher and approached the ecological limits faced by *M. reukaufii* in the wild; (ii) the alterations in DNA methylation profiles experienced by individual genotypes following exposure to variable sugar environments were adaptive, in the sense that they allowed genotypes to exploit the most extreme sugar environments; and (iii) the broad population niche width of *M. reukaufii* in our study area was largely a consequence of broad plasticity in resource use by individual genotypes arising from environmentally induced epigenetic changes in DNA methylation. Homogeneity of strains in their epigenetic response to experimental treatments supports this interpretation.

The molecular mechanisms responsible for the association between methylation of certain zones of the genome of *M. reukaufii* and this yeast's ability to grow in extreme sugar environments can only be tentatively guessed at present. In plants, DNA methylation controls gene expression through interfering with transcription and influencing the formation of transcriptionally silent heterochromatin (Finnegan *et al.* 1998; Zilberman *et al.* 2007; Jablonka & Raz 2009). Similar functions have been also proposed for DNA methylation in fungi, and specific genes whose expression is regulated via methylation have been identified (Tamame *et al.* 1983b, 1988; Magill & Magill 1989; Martienssen & Colot 2001). The methylation-dependent ability of *M. reukaufii* to proliferate in hexose-dominated media at high sugar concentrations might perhaps be related to methylation-mediated regulation of hexose transporter (*Hxt*) genes or others involved in the synthesis of the 'compatible solutes' that protect cells from high osmotic pressures (Özcan & Johnston 1999; Grant 2004).

Epigenetics and phenotypic plasticity

Although phenotypic plasticity is central to the survival of all organisms, it is expected to be particularly important in those where a limited control of spatial position restricts their capacity to actively select the features of their immediate surroundings. These organisms have to cope with the consequences of passively colonizing habitat patches whose characteristics may vary widely in time or space and are therefore expected to exhibit broad phenotypic plasticity. It is not surprising, therefore, that plants and fungi are notable for their phenotypic plasticity (Herrera 2009; Slepecky & Starmer 2009). Results presented here for the specialized nectar-living yeast *M. reukaufii* confirm theoretical expectations that broad plasticity should evolve in regularly colonizing species with a metapopulation structure that exploit fine-grained habitats where environmental factors vary over small spatial scales (Roughgarden 1972; Sultan & Spencer 2002; Baythavong 2011). Although the few genotypes used in our experiments did not allow for partitioning resource use by *M. reukaufii* into between- and within-genotype components, all strains were similar in their DNA methylation responses to variable sugar environments. Our results thus provide one of the clearest examples to date supporting the classical prediction that broad population niche widths in fugitive species should mainly stem from an ability of individual genotypes to exploit broad resource ranges thanks to their extensive phenotypic plasticity (Roughgarden 1972).

As noted in the Introduction, difficulties involved in obtaining sufficient replicates with identical genotypes has hindered progress in understanding the mechanisms and consequences of single-genotype responses to variable environments for sexually reproducing multicellular organisms that cannot be easily cloned for experimentation. The natural clonal life style of *M. reukaufii* was thus particularly helpful to attain reliable conclusions on the epigenetic mechanisms underlying plasticity, an advantage that make unicellular fungi and, more generally, clonal microbes particularly valuable subjects for experimental studies of niche width (Kassen 2002; Slepecky & Starmer 2009). Natural variation in gene methylation can be under complex genetic and epigenetic control (Zhang 2008; Jablonka & Raz 2009), and establishing the degree to which epigenetic variation is autonomous from genetic variation is

central to evaluating the evolutionary relevance of the former as an additional inheritance system (Richards 2006; Bossdorf *et al.* 2008; Jablonka & Raz 2009). The multiplication of single genotypes in *M. reukaufii* allowed us to identify adaptive within-genotype DNA methylation polymorphisms that took place in absence of any genetic (i.e. sequence-based) variation. This finding has obvious implications for the enduring debate in evolutionary biology on the relative importance of structural vs. regulatory variation in adaptive evolution (e.g. Durbin *et al.* 2003). As shown here, non-structural, presumably regulatory variation alone can under certain circumstances play a key adaptive role in enhancing the fitness of genotypes and allowing the local persistence of populations by broadening the range of exploitable resources. This leads to the corollary that plasticity based on variable DNA methylation (i.e. the ability to adjust epigenotype within a single generation, as shown here) might, under such circumstances, be subject to selection in and of itself, which would in turn provide the raw material and a starting pathway for the perpetuation in populations of adaptive, heritable, evolutionarily relevant epigenetic variation (i.e. the transgenerational persistence of environmentally changed epigenotypes; Jablonka & Raz 2009; Verhoeven *et al.* 2010). Another aspect deserving consideration is that, because of its lability, epigenetic change endows genotypes with a highly flexible plasticity mechanism which may protect them from the negative genetic correlations in fitness across environments traditionally assumed in models of niche width evolution (Levins 1968; Futuyma & Moreno 1988). Epigenetically regulated plasticity may thus allow the evolution of jacks of all trades that are masters of many, as shown here for *M. reukaufii.*

Acknowledgements

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Data accessibility

Nectar concentration and composition data, and colony growth data and methylation-sensitive amplified polymorphism (MSAP) fingerprints of *Metschnikowia reukaufii* strains cultured under control and experimental conditions are deposited at DRYAD entry doi: 10.5061/dryad.k89b2ps8.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Insect-pollinated plants with floral nectar inhabited by *Metschnikowia reukaufii* in the study region.

Table S2 Flower visits recorded and identity of main pollinators for focal host plants of *Metschnikowia reukaufii* included in the study of pollinator sharing.

Fig. S1 Pairwise similarity in pollinator composition between representative host plants of *Metschnikowia reukaufii* flowering at different times along the blooming season.

Fig. S2 Mean growth index of *Metschnikowia reukaufii* in experimental media differing in sugar composition, sugar concentration, and treatment with the DNA methylation inhibitor 5-azacytidine.

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