

Effects of gene flow on phenotype matching between two varieties of Joshua tree (*Yucca brevifolia*; Agavaceae) and their pollinators

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Abstract

In animal-pollinated plants, local adaptation to pollinator behaviour or morphology can restrict gene flow among plant populations; but gene flow may also prevent divergent adaptation. Here, we examine possible effects of gene flow on plant–pollinator trait matching in two varieties of Joshua tree (Agavaceae: *Yucca brevifolia*). The two varieties differ in strikingly in floral morphology, which matches differences in the morphology of their pollinators. However, this codivergence is not present at a smaller scale: within the two varieties of Joshua tree, variation in floral morphology between demes is not correlated with differences in moth morphology. We use population genetic data for Joshua tree and its pollinators to test the hypotheses that gene flow between Joshua tree populations is structured by pollinator specificity, and that gene flow within the divergent plant–pollinator associations ‘swamps’ fine-scale coadaptation. Our data show that Joshua tree populations are structured by pollinator association, but the two tree varieties are only weakly isolated – meaning that their phenotypic differences are maintained in the face of significant gene flow. Coalescent analysis of gene flow between the two Joshua tree types suggests that it may be shaped by asymmetric pollinator specificity, which has been observed in a narrow zone of sympatry. Finally, we find evidence suggesting that gene flow among Joshua tree sites may shape floral morphology within one plant–pollinator association, but not the other.

Introduction

The efficiency, specificity and dispersal ability of animal pollinators directly shape gene flow between populations of the plants that rely on them. Pollinators are therefore thought to be a major source of reproductive isolation among flowering plant populations (Grant, 1949; Kiestler *et al.*, 1984; Coyne & Orr, 2004), which may explain increased rates of species formation associated with more specialized pollination systems

(Hodges & Arnold, 1995; Sargent, 2004; Armbruster & Muchhala, 2009). Pollinators may help to create reproductive isolation in two major ways. First, if pollinators specialize on flowers that provide the most benefits, they may transfer pollen only among most beneficial subset of host populations (Campbell *et al.*, 1997; Kay, 2006; Martin *et al.*, 2008; Dell’Oliveo *et al.*, 2011). Second, selection for more efficient pollen transfer may favour plants whose flowers match their pollinators’ morphology, leading to morphological divergence between plant populations served by alternative pollinators (Aigner, 2005; Anderson & Johnson, 2007; but see Aigner, 2004). These two sources of reproductive isolation and evolutionary divergence

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may often work synergistically; reduced pollen transfer between plant populations should facilitate morphological divergence, which may in turn further reduce pollen transfer.

Multiple lines of evidence suggest that pollinator interactions have some role in angiosperm speciation. Fossil evidence demonstrates the synchronous diversification of pollinators and angiosperms over geological timescales (Grimaldi, 1999), phylogenetic analyses have found that floral traits facilitating more specific pollinator interactions are associated with increased net rates of species formation (Hodges & Arnold, 1995; Dodd *et al.*, 1999; Sargent, 2004), and ecological studies have firmly established the importance of pollinator specificity in maintaining reproductive isolation between species (Grant, 1949; Campbell *et al.*, 1997; Hodges *et al.*, 2003; Ramsey *et al.*, 2003; Martin *et al.*, 2008; reviewed in Coyne & Orr, 2004). However, it is not clear how often pollinator-mediated isolation *arises* as a result of the plant–pollinator interaction – that is, how often the dynamics of coevolution within plant–pollinator associations actually contributes to speciation of the associates. One of the most widely cited theoretical treatments of plant–pollinator codivergence, by Kiester *et al.* (1984), requires one species to diverge as a result of drift, sexual selection or adaptation to the abiotic environment before a plant–pollinator interaction can create codivergence. More recent analysis of plant–pollinator coevolution shows that, rather than promoting greater divergence, mutualism may often reduce phenotypic diversity within populations (Kopp & Gavrillets, 2006) and among demes in spatially structured habitats (Yoder & Nuismer, 2010). These results are consistent with the possibility that pollinator specialization does not create greater plant diversity, but rather that diversity creates specialization, via stronger competition for pollinators within more diverse plant groups (Armbruster & Muchhala, 2009).

The interaction between Joshua trees (*Yucca brevifolia* Englem.; Agavaceae) and the moths that pollinate them (*Tegeticula* spp.; Prodoxidae) offers the chance to observe plant–pollinator diversification at close range. Joshua trees and their pollinators are engaged in the classic obligate mutualism of yuccas and yucca moths. Female yucca moths carry pollen to Joshua tree flowers in uniquely derived specialized mouthparts; inject eggs into the floral ovaries using a sclerotized, bladelike ovipositor; and then actively apply pollen to the stigmatic surface to fertilize the flower (Trelease, 1892; Pellmyr, 2003). As a Joshua tree flower develops into a fruit, the moth eggs hatch and the emerging larvae eat a portion of the developing seeds. Yucca moths are the sole pollinators of yuccas, and yucca seeds the only food source for the moths (Riley, 1873; Pellmyr, 2003). The interaction is stabilized by a selective floral abortion mechanism, in which yuccas abscise flowers that receive too many moth eggs or too little pollen (Pellmyr

& Huth, 1994; Huth & Pellmyr, 2000; Marr & Pellmyr, 2003).

In Joshua tree, this mutualism is thought to have facilitated the codivergence of the plant and its pollinators. Joshua tree populations are pollinated by one of two yucca moths, *Tegeticula synthetica* Riley or *T. antithetica* Pellmyr, sister species whose common ancestor probably used Joshua tree (Pellmyr & Segraves, 2003; Godsoe *et al.*, 2008; Smith *et al.*, 2008). The two pollinator species are codistributed with two parapatric varieties of Joshua tree: *Yucca brevifolia* var. *brevifolia* in the western portion of the Mojave desert and *Y. brevifolia* var. *jaegeriana* in the eastern portion, with a small area of sympatry at a ~5-km-wide region in central Nevada, Tikaboo Valley (Fig. 1a,b; Rowlands, 1978; Smith *et al.*, 2009).

The two varieties of Joshua tree were originally distinguished by differences in vegetative morphology (Rowlands, 1978), but investigations with reference to the pollinator species revealed that their flowers are still more different, particularly in the length of the stylar canal. This measurement approximates the distance a female moth's ovipositor must extend to deposit eggs (Fig. 1c; Godsoe *et al.*, 2010), and matches almost exactly the difference in body size between the two moth species (Fig. 1c). These parallel differences in stylar canal length and moth ovipositor length support the hypothesis that the floral abortion mechanism selects for plant–pollinator phenotype matching (Godsoe *et al.*, 2008). The two varieties of Joshua tree occupy environments with essentially similar climatic conditions (Godsoe *et al.*, 2009), further suggesting that selection exerted by their pollinators is the best explanation for the morphological divergence of the trees.

Observations by Smith *et al.* (2009) at the Tikaboo Valley contact zone, where both moth species encounter both tree varieties, reveal a more nuanced picture of asymmetric pollinator specificity. *Yucca brevifolia* var. *jaegeriana* trees were more frequently visited by *T. antithetica* adults than by *T. synthetica*, but the two pollinator species were captured with almost equal frequency on *Y. b. brevifolia*. However, the two moth species had strikingly different success in rearing larvae on the two tree varieties. Female *T. antithetica* ovipositing on 'foreign' *Y. b. brevifolia* trees had fewer surviving offspring than those ovipositing on *Y. b. jaegeriana*, and no female *T. synthetica* successfully reared larvae on *Y. b. jaegeriana* (Smith *et al.*, 2009). A subsequent study in Tikaboo Valley used genetic markers to identify recent hybrids between the two tree varieties, and found that while hybrid trees are widespread in the contact zone, there is significantly greater gene flow from *Y. b. jaegeriana* into *Y. b. brevifolia* than *vice versa* – consistent with the lower host fidelity seen in *T. antithetica* (Starr *et al.*, 2013).

These observations of ongoing hybridization between *Y. b. brevifolia* and *Y. b. jaegeriana* suggest that their phenotypic differences are maintained against the

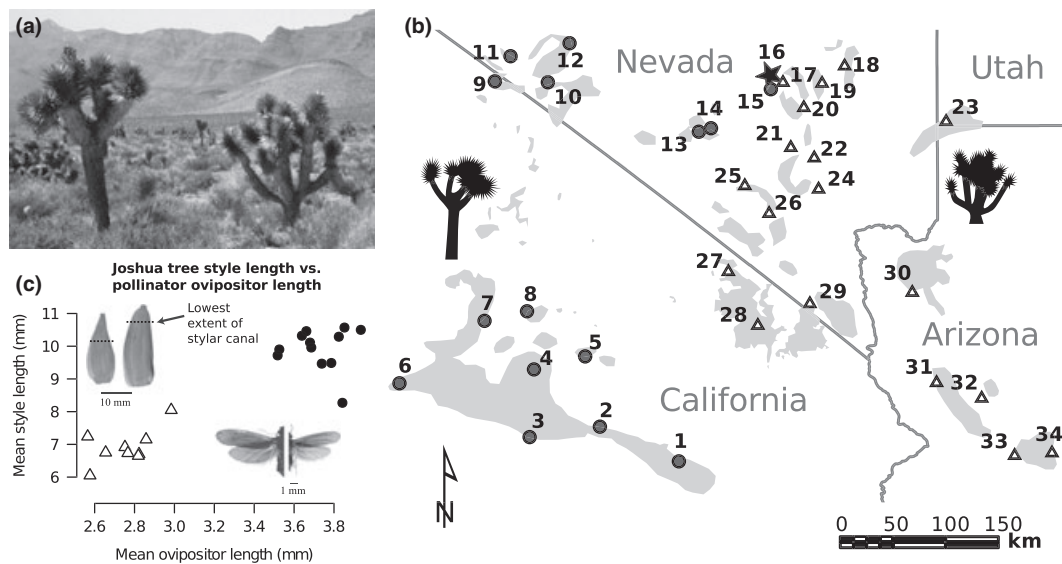


Fig. 1 The two varieties of Joshua tree and their pollinators. (a) *Yucca brevifolia* var. *brevifolia* (left) growing next to *Y. brevifolia* var. *jaegeriana* at Tikaboo Valley, Nevada. (b) Sites sampled for this study. The range of Joshua tree is shaded. Dark grey dots are sites occupied by var. *brevifolia* and *Tegeticula synthetica*; white triangles are sites occupied by var. *jaegeriana* and *T. antithetica*; the black star is Tikaboo Valley, Nevada, where both tree varieties and both moth species are found. Site numbers follow Table 1. (c) Scatter plot of site mean style length for Joshua tree vs. site mean ovipositor length for its pollinators, with points coloured according to pollinator species and tree type as in (b), with cross-sectional images of representative floral ovaries and half-images of pinned female moths. Data for (c) from Godsoe *et al.* (2010); see also Table 1.

homogenizing effects of gene flow by selection from the interaction with pollinators (Smith *et al.*, 2009; Starr *et al.*, 2013). Such an interplay of gene flow with local adaptation to interacting species has been a major focus of empirical and theoretical studies of coevolution, as synthesized in the geographical mosaic theory of coevolution (Thompson, 1994, 2005, 2009), which suggests that immigration may often ‘swamp’ local adaptation, preventing fine-scale coadaptation of interacting species (Nuismer *et al.*, 1999; Thompson, 1999, 2005; Gomulkiewicz *et al.*, 2003, 2007).

Indeed, although the codivergence of Joshua tree and its pollinators is striking at the level of differences between the two Joshua tree varieties, a study of phenotype variation *within* the two tree varieties found no such pattern of phenotype matching at the finer scale (Fig. 1c; Godsoe *et al.*, 2010). The absence of phenotype matching on a local scale might be explained by gene flow among populations of trees and moths if the magnitude of gene flow is great enough to overcome the effects of local coevolution (Slatkin, 1973, 1987; Nuismer *et al.*, 1999; Morgan *et al.*, 2005). In that case, local plant–pollinator coevolution may have the effect of creating closer matching, but adaptive change in one or both species is offset by immigration from other sites with different, maladaptive phenotypes. It may also mean that coevolutionary selection on a local scale is weak relative to selection against interaction with the ‘foreign’ partner. That is, the adaptive landscape arising from the plant–pollinator

interaction does not have two separate peaks, so much as two separate plateaus, across which local populations are free to vary as a result of drift or adaptation to the environment without compromising the functioning of the pollination mutualism. Both these hypotheses have been proposed as (nonexclusive) explanations for the observed pattern (Godsoe *et al.*, 2010), but population genetic data were not available to test whether gene flow within the two varieties of Joshua tree prevents closer coevolutionary matching.

Here, we use data from recently developed nuclear microsatellite markers for Joshua tree (Flatz *et al.*, 2011) and its pollinators (Drummond *et al.*, 2009a; Smith *et al.*, 2009) to test the hypotheses that pollinator specificity and movement shape gene flow within and between the two varieties of Joshua tree, and that gene flow between demes prevents closer phenotype matching between Joshua trees and their pollinators on a population scale. First, we examine whether and how Joshua tree populations are isolated by pollinator association, using analysis of molecular variance (AMOVA), Bayesian clustering and coalescent simulation. We then test whether the population genetic structure of Joshua tree is related to the population genetic structure in its pollinators. Next, we use the microsatellite data to re-examine population-level morphological variation in Joshua tree and its pollinators (Godsoe *et al.*, 2008, 2010), and determine whether floral differences between the two tree varieties are better explained by differences in pollinator ovipositor

Table 1 Sampling for this study.

ID*	Site name	Sample size for		Moth microsatellites [‡]	Moth ovipositor [†]	Lat. (°N)	Lon. (°W)
		Tree microsatellites	Tree style [†]				
<i>Tegeticula synthetica</i> (western) sites [§]							
1	Joshua Tree	15	8	10	2	34.1368	116.1604
2	San Andreas	8	10		3	34.4762	116.8069
3	Freemont Peak	15		5		34.2363	117.4855
4	Kramer Hills	15	10	17	5	34.9030	117.4853
5	Fossil Bed Road		9	6	5	35.0033	117.0339
6	Lancaster-Palmdale		10	8	1	34.7733	118.5039
7	Cactus Flats	13	10	5	6	36.2113	117.9352
8	Trona	15	10	4	2	35.4757	117.5869
9	Eureka Valley	15	10	8	9	37.3790	117.8277
10	Gold Point	10	10	5	1	37.3589	117.3697
11	Clayton Valley	8		5	1	37.5833	117.7333
12	Montezuma Peak	9		3	1	37.7407	117.2616
13	Nevada Test Site		10	8	4	36.9599	116.0681
14	Nevada Test Site II	16	10	6	3	36.9794	115.9895
<i>Contact zone</i>							
15	Tikaboo South	16		2		37.3744	115.5142
16	Tikaboo Valley	75	26	20		37.4816	115.5060
17	Hancock Summit	12	10			37.4158	115.3901
<i>T. antithetica</i> (eastern) sites							
18	Delamar Valley	15	8	5	3	37.5249	114.8158
19	Eightmile Valley	10	10	6	5	37.3740	115.0304
20	Sheep Range	4	6		5	37.1783	115.1852
21	Sheep Pass	3	10	5		36.8292	115.2988
22	Sawmill Road		10		4	36.7068	115.0423
23	Shivwitts	12		10	4	37.0638	113.8969
24	Yucca Forest	15	10	6		36.4781	115.0577
25	Indian Ridge	9	10	4	1	36.5007	115.6897
26	Kyle Canyon		4	4		36.2667	115.5150
27	Kingston Mountains	14	10	11	2	35.7673	115.8414
28	Cima	15		5		35.3638	115.5887
29	Searchlight	15	6	4	2	35.5201	115.1405
30	Dolan Springs	15	9	2	1	35.5982	114.2600
31	Alamo Crossing		6		8	34.8062	114.0738
32	Wikieup	15	10	18		34.6803	113.6921
33	Joshua Tree Parkway	14	10	8		34.2258	113.0736
34	Alamo Lake	10	2	4	2	34.1998	113.3933

*Site ID corresponds to numbers in Fig. 1(b).

[†]Morphological data originally published by Godsoe *et al.* (2010).

[‡]Moth microsatellite data originally published by Drummond *et al.* (2009a).

[§]Site groups based on the results of Structure clustering, and as organized for *Migrate-n* analysis.

length or by neutral genetic variation – the former being consistent with the hypothesis that moths exert divergent selection on Joshua tree flowers, and the latter with the hypothesis that phenotypic differences are simply due to genetic drift.

We find that Joshua tree populations are structured by pollinator association, but that there is substantial ongoing gene flow between the two subspecies of Joshua tree. That their phenotypic differences persist in the face of gene flow suggests that this divergence may be maintained by pollinator-mediated selection. We also find indications of contrasting effects of gene

flow on style length in the two different varieties of Joshua tree: for *Y. b. jaegeriana*, style length may be explained by gene flow and pollinator phenotypes, but for *Y. b. brevifolia*, our data suggest pollinator population structure is the best predictor of Joshua tree phenotypes.

Materials and methods

Sample collection, DNA extraction and genotyping

For our population genetic analysis of Joshua tree and its pollinators, we sampled 34 sites across the tree's

range: 15 sites pollinated exclusively by *Tegeticula synthetica*, 18 pollinated exclusively by *T. antithetica* and the pollinator/tree variety contact zone in Tikaboo Valley, Nevada (Fig. 1, Table 1). At each site, we sampled trees by taking leaf tissue clippings of less than 5 g from up to 20 haphazardly selected individuals. We stored collected plant tissue in liquid nitrogen until samples could be returned to the laboratory, where they were kept at -80°C until use. We extracted total genomic DNA using DNEasy Plant Mini Kits (Qiagen, Inc; Valencia, CA, USA), first disrupting the tissue under liquid nitrogen and then following the standard kit protocols. We quantified the total nucleic acid produced by each extraction using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and created working stocks at concentrations between 15 and $35\text{ ng }\mu\text{L}^{-1}$. Undiluted extractions were stored at -20°C , and working stocks at 4°C while in regular use.

We used the working genomic stocks to genotype each sampled Joshua tree individual at 10 microsatellite loci for Joshua tree, following multiplex PCR protocols published by Flatz *et al.* (2011) and using fluorescent dye-tagged primers. Although Flatz *et al.* (2011) describe 12 microsatellite loci, we excluded one locus in which null alleles were found (Yucbre05) and one whose repeat pattern rendered allele binning problematic (Yucbre20). We determined the length of fragments in multiplex PCR products by capillary electrophoresis on an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, CA, USA), and developed binning schemes for the fragment profiles from each locus based on saturation plots of the raw fragment sizes. A subsample of individuals were genotyped multiple times to confirm binning and genotyping consistency.

The final Joshua tree genetic data set contains complete 10-locus genotypes for 408 individuals (Table 1). We report expected and observed heterozygosity and results of a test for heterozygote deficiency for each locus at each sampling site, as calculated and estimated using GenePop (version 4.0.10, implemented online at <http://genepop.curtin.edu.au>), in Table S1 in the Supporting Information. We excluded the loci Yucbre08, Yucbre22 and Msat-36 from further analysis because they showed statistically significant deviation from Hardy–Weinberg equilibrium at more than five sampling sites (Table S1).

The range-wide pollinator microsatellite data set was previously published as a reference population for identifying the species of moth larvae extracted from fruit in the Tikaboo Valley contact zone (Smith *et al.*, 2009); but it has not previously been used to study population genetic structure within the pollinator species. This data set consists of genotypes for 227 individuals (Table 1; 102 *T. synthetica* and 125 *T. antithetica*), 194 of which are complete at nine microsatellite loci (Drummond *et al.*, 2009a). Methods for sample collection, genomic DNA extraction and microsatellite genotyping are described by Drummond *et al.* (2009a) and Smith *et al.* (2009).

Plant and pollinator phenotype data

To test for the effects of plant and pollinator gene flow on plant–pollinator trait matching, we re-examined previously reported phenotype data sets for Joshua tree (Godsoe *et al.*, 2008) and its pollinators (Godsoe *et al.*, 2010); specimen collection and measurement methods are reported in these respective original sources. Of the Joshua tree phenotype measurements reported by Godsoe *et al.* (2008), we focus solely on the length of the stylar canal, which most strongly differentiates the two Joshua tree varieties and is most directly involved in the success or failure of pollinator oviposition. Prior to analysis, we rechecked the original records for all phenotype measurements, and, in the case of Joshua tree style lengths, conducted additional measurements on new flowers from two collection sites. In the end, we retained all phenotype data except for floral measurements from Shivwits (site 23, Table 1 and Fig. 1b), which we determined had been taken from immature flowers.

Population genetic structure of Joshua tree and its pollinators

We tested for genetic structure in Joshua tree and pollinator populations at the level of collection site and pollinator association (for the trees) or species (for the pollinators) with AMOVA implemented in the program *GenoDive* (version 2.0b17; Meirmans & Van Tienderen, 2004), using the stepwise mutation model to estimate Φ_{ST} statistics (analogous to F_{ST}), and testing for the significance of estimated parameters by comparison with estimates from 10^4 permutations of the data set. Because trees in the Tikaboo Valley contact zone could not be clearly assigned to one pollinator association *a priori*, we excluded them from the AMOVA.

We also tested for population structure in Joshua tree using the Bayesian clustering algorithm implemented using the program *Structure* (version 2.31; Pritchard *et al.*, 2000; Falush *et al.*, 2003, 2007). We ran five replicate *Structure* analyses each for values of K , the number of genotype clusters, ranging from 1 to 14. We then used the ΔK statistic Evanno *et al.*, 2005 to identify the K value best supported by our data from the results of the replicate runs. For all *Structure* runs, we ran the Markov chain Monte Carlo (MCMC) algorithm for 2×10^5 iterations preceded by 5×10^4 iterations of burn-in, using collection sites as prior information for the clustering algorithm, with admixture and correlated gene frequencies among genotype clusters assumed.

Finally, we estimated bidirectional rates of gene flow between the contact zone and ‘pure’ groupings of Joshua tree sites using the coalescent analysis implemented using the program *Migrate-n* (version 3.2.19; Beerli & Felsenstein, 1999, 2001; Beerli, 2006). We ran the program in parallel mode on the supercomputing

cluster maintained by the Minnesota Supercomputing Institute (MSI) at the University of Minnesota.

On the basis of the results of the *Structure* analysis, we divided collection sites into three groupings: 'pure' western and eastern sites associated exclusively with one or the other pollinator species, and the contact zone, consisting of the Tikaboo Valley, Tikaboo Valley South and Hancock Summit sites (Table 1). We set *Migrate-n* to use the Brownian motion approximation of a ladder model of mutation, allowing different mutation rates for each locus. We used the Bayesian inference mode, determining appropriate prior distributions for estimated parameters by adjusting the priors over repeated runs until posterior distributions for each parameter showed no evidence of truncation by the prior distribution. For the final analysis, we set the algorithm to draw values for the mutation-scaled population size (θ , equal to $4 \times N \times \mu$ in diploids) from a uniform prior distribution between 0 and 100; and values for the mutation-scaled rate of migration (M , equal to m/μ) from a uniform prior distribution between 0 and 1000. We assessed the convergence of the algorithm on parameter estimates by inspection of the posterior distributions for each parameter, and stationarity based on the effective sample size (ESS) estimated by *Migrate-n*. We reiterated estimation with increasing chain length until convergence and stationarity were achieved. A preliminary run estimating migration between all possible pairs of population groupings returned estimates of direct migration between pure *Y. b. brevifolia* and *Y. b. jaegeriana* populations with 95% confidence intervals (CI) that overlapped zero; so for our final estimation, we restricted direct migration between the two pure regions to zero – so that all migration between the two tree varieties was mediated by the Tikaboo Valley contact zone. For final parameter estimation, we set the algorithm to take 5×10^3 samples at intervals of 500 steps after 5×10^4 steps of burn-in.

Pollinator isolation among Joshua tree populations

We then used the microsatellite data sets for both Joshua tree and its pollinators to test the hypothesis that pollinator specificity and migration patterns shape rates of gene flow among Joshua tree populations. We tested this hypothesis using a series of interrelated matrix correspondence tests (MCT, or Mantel tests; Mantel, 1967; Smouse *et al.*, 1986), which we conducted in the statistical computing environment R (version 2.14.1 R Core Team, 2012). First, we tested for significant effects of isolation by distance (IBD; Wright, 1943; Rousset, 1997), in the Joshua tree and pollinator microsatellite data, then tested to see whether IBD was stronger between Joshua tree sites pollinated by different moth species than between sites pollinated by the same moth species (i.e. pollinator isolation).

Isolation by distance in Joshua tree and its pollinators

For all MCTs, we used pairwise, standardized Φ_{ST} calculated in *GenoDive* (Hedrick, 2005; Meirmans, 2006) as a measure of genetic distance between sampling sites. To test for IBD (Wright, 1943; Rousset, 1997), we used *R* to perform a regression between $\Phi_{ST}/(1-\Phi_{ST})$ and the logarithm of geographical distance between sampling sites (following Rousset, 1997); we tested the significance of this relationship using a MCT. We also tested for an association between Joshua tree genetic distance and pollinator genetic distance between collection sites, which would indicate that pollinator-mediated gene flow is a major determinant of Joshua tree population structure.

Joshua tree population genetics and pollinator population structure

If pollinator isolation amplifies isolation by distance among Joshua tree sites, the slope of the IBD relationship should be greater for distances between collection sites pollinated by different moth species than the slope of the relationship for distances between sites pollinated by the same moth species. That is, the genetic distance between two sites should increase more rapidly with geographical distance if those two sites are pollinated by different moth species than if they are pollinated by the same moth species. We tested for this effect by performing separate regressions of genetic distance on geographical distance between (1) pairs of sites pollinated by the same moth species and (2) pairs of sites pollinated by different moth species. We tested the hypothesis that the difference in the slopes of these two regression lines was greater than expected by chance by comparing the observed difference with the distribution of differences obtained by randomly permuting the data set into two groupings and recalculating regression slopes.

Testing for gene flow swamping local plant–pollinator phenotype matching

If plant–pollinator phenotype matching in Joshua tree is prevented by gene flow among different Joshua tree populations, then a significant relationship between Joshua tree style length and moth ovipositor length may become apparent only after controlling for genetic distances between collection sites. We therefore used the morphological data set to test for correlations between Joshua tree and pollinator traits, with the Joshua tree and yucca moth microsatellite data as a covariate, to test whether the relationship between Joshua tree floral style length and pollinator ovipositor length is greater than expected, given neutral genetic differences between sampling sites.

A key result of the original analysis by Godsoe *et al.* (2010) is that the correlation between Joshua tree style length and moth ovipositor length is strong when

comparing across pollinator species and tree varieties, but undetectable within pollinator species and tree varieties. We therefore tested for a trait-matching relationship between Joshua tree style length and pollinator ovipositor length, both across all sites (i.e. encompassing both tree varieties) and within each association of Joshua tree variety and moth species. To accomplish this, we summarized the genetic differences among Joshua tree and moth populations using principal components analysis (PCA) on the matrix of pairwise genetic distances between Joshua tree and moth collection sites, then used the first principal component from each as a covariate in a multiple linear regression framework. For Joshua tree, the first principal component axis captures 53% of the variation in the pairwise genetic distance matrix, and larger values along this axis roughly correspond to eastern *Y. b. var. jaegeriana* populations and smaller values correspond to western *Y. b. var. brevifolia*; for the pollinators, the first PC axis captures 75% of variation in the pairwise genetic distance matrix, with larger values along the axis corresponding to *T. antithetica* populations, and smaller values to *T. synthetica*.

With these summaries of neutral genetic identity for each site, we tested for an association between plant and pollinator population genetics, which would support the hypothesis that pollinator-mediated gene flow shapes neutral genetic variation among Joshua tree populations. Using native functions in *R*, we tested for correlations between plant and pollinator genetics using both the range-wide data set, and within the subsets of data for western *Y. b. brevifolia* and eastern *Y. b. jaegeriana* sites.

We then compared the goodness-of-fit for linear models in which moth ovipositor length, Joshua tree genetics, moth genetics, moth species identity or combinations of those variables were used as predictors for Joshua tree style length. Because we anticipate, from the results of Godsoe *et al.*, that different patterns may be apparent across all Joshua tree populations and within the two separate plant–pollinator associations, we fit the models using (1) the complete range-wide data set, (2) data from only the western sites and (3) data from only the eastern sites. If gene flow among Joshua tree populations swamps local adaptation to pollinators, then models incorporating Joshua tree genetics should better explain variation in Joshua tree style length than models without Joshua tree genetics as a covariate. Also, if pollinator-mediated gene flow is responsible for ‘swamping’ plant–pollinator trait matching, models incorporating pollinator genetic data should better explain style length than those that do not. We performed this analysis in *R*, and compared the goodness-of-fit for candidate models based on their Akaike’s Information Criterion scores, corrected for sample size (AICc; Burnham & Anderson, 2004).

Results

Population genetic structure of Joshua tree and its pollinators

Analysis of molecular variance

AMOVA results for the Joshua tree microsatellite data set are presented in Table 2. The majority of variation observed is within individuals (59.0% of variation), which reflects the high variability of the microsatellite loci (Fitz *et al.*, 2011). The percentage of observed variation partitioned among sampling sites and pollinator associations is low: 8.4% and 15.1% respectively (Table 2).

AMOVA results for the pollinator microsatellite data confirm previous analyses, which have found strong isolation between *T. synthetica* and *T. antithetica* (Smith *et al.*, 2008), but relatively little structure within each species (Drummond *et al.*, 2009a). Substantially more variation is partitioned among pollinator species than among collection sites (41.5% vs. 1.2%; Table 3).

Bayesian clustering analysis

In *Structure* runs assuming $K = 2$ genotype clusters, many trees in our data set were ambiguously assigned (i.e. the probability of assignment is between 0.75 and 0.25), particularly at the Tikaboo Valley contact zone (Fig. 2, upper panel). This is consistent with direct evidence of ongoing hybridization between Joshua tree varieties (Smith *et al.*, 2009; Starr *et al.*, 2013). Our replicate runs of *Structure* across multiple possible values for K found the strongest support for $K = 3$ ($\Delta K = 15.51$ for $K = 3$ compared with 3.97 for the next-best value; Evanno *et al.*, 2005). The plot of *Structure*’s probability of assignment to each of the three clusters for each tree in our data set shows that two clusters largely correspond to the ‘pure’ populations of two Joshua tree varieties

Table 2 Analysis of molecular variance for the Joshua tree microsatellite data.

Source of variation	Nested in	%var.	Φ_{xx}	Φ -value	SE	$P(\Phi_{xx})$
Within individual	–	59.0	Φ_{IT}	0.410	0.068	–
Among individual	Site	17.4	Φ_{IS}	0.228	0.089	0.000
Among site	Pollinator	8.4	Φ_{SC}	0.100	0.017	0.000
Among pollinator	–	15.1	Φ_{CT}	0.151	0.072	0.000

Table 3 Analysis of molecular variance for moth microsatellite data.

Source of variation	Nested in	%var.	Φ_{xx}	Φ -value	SE	$P(\Phi_{xx})$
Within individual	–	49.3	Φ_{IT}	0.507	0.058	–
Among individual	Site	8.0	Φ_{IS}	0.140	0.151	0.004
Among site	Species	1.2	Φ_{SC}	0.021	0.007	0.151
Among species	–	41.5	Φ_{CT}	0.415	0.081	0.000

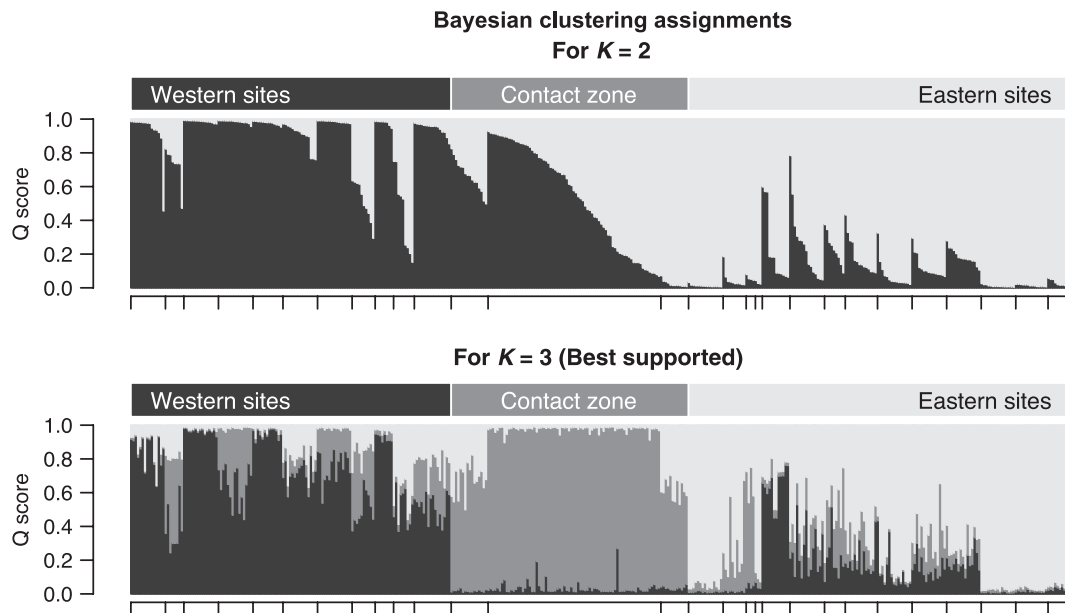


Fig. 2 Bayesian clustering of Joshua tree microsatellite genotypes. Each vertical bar represents a single genotype, coloured proportionally to its probability of assignment to each possible cluster. Genotypes are arrayed within sampling sites from west to east in the order sites are numbered in Fig. 1 and Table 2. Top panel: clustering assignments for $K = 2$. Bottom panel: clustering assignments for $K = 3$, the best supported number of clusters according to the ΔK test of Evanno *et al.* (2005), and which highlights recent admixture at Tikaboo Valley and adjacent sites.

and their pollinator associations, and the third to the Tikaboo Valley contact zone and immediately adjacent sites; this third cluster may reflect the region of most recent genetic admixture between *Yucca brevifolia* var. *brevifolia* and *Y. b. var. jaegeriana* (Fig. 2, lower panel).

Joshua tree population structure and pollinator isolation

Isolation by distance

We found a statistically significant relationship between genetic distance and geographical distance for Joshua tree as a whole (Mantel $P = 3 \times 10^{-4}$). However, the two varieties of Joshua tree showed different patterns of isolation by distance (Fig. 3a): there was a significant positive relationship between genetic distance and geographical distance in *Y. b. brevifolia* ($P = 0.0241$), but no such relationship in *Y. b. jaegeriana* ($P = 0.5382$). Isolation by distance was also stronger (i.e. genetic distance increased more rapidly with geographical distance) for pairs of sampling sites pollinated by different moth species than for pairs of sites pollinated by the same species (Fig. 3b), and our permutations of the data set found that the observed difference in regression slopes was greater than expected by chance ($P < 10^{-4}$). Pairwise MCTs found no significant relationship between genetic distance and geographical distance in *T. synthetica* ($P = 0.1852$; Fig. 3c), and a weak, marginally significant relationship in *T. antithetica* ($P = 0.0686$; Fig. 3c).

Asymmetric gene flow estimates

We report all Bayesian posterior parameter estimates and their 95% CI for mutation-scaled population size and effective rates of immigration and emigration per generation (as m/μ) between populations in Table 4. The posterior probability distributions for all parameters are clearly unimodal, suggesting reasonably good convergence on parameter estimates (Fig. S1), and *Migrate-n* reports an effective sample size ≥ 2000 for all parameters, which we take to indicate that the algorithm sampled from a stationary posterior probability distribution.

In terms of migrants per generation (Nm ; Table 4), mean estimated gene flow rates are substantially in excess of the minimum typically understood to prevent neutral genetic differentiation, one migrant per generation (Wright, 1940); and mutation-scaled migration rates M revealed large contributions of genetic variation into Tikaboo Valley from both western and eastern Joshua tree populations. However, whereas the 95% CI of the mutation-scaled migration rate from the contact zone into western populations overlaps with zero, our analysis found that the rate from Tikaboo Valley into eastern populations is significantly greater than zero (Table 4, Fig. S1). This is consistent with previous observations that *T. antithetica* exhibits lower host fidelity in the contact zone (Smith *et al.*, 2009) and population genetic evidence that gene flow from hybrids to *Y. b. jaegeriana* is more common than from hybrids to *Y. b. brevifolia* (Starr *et al.*, 2013).

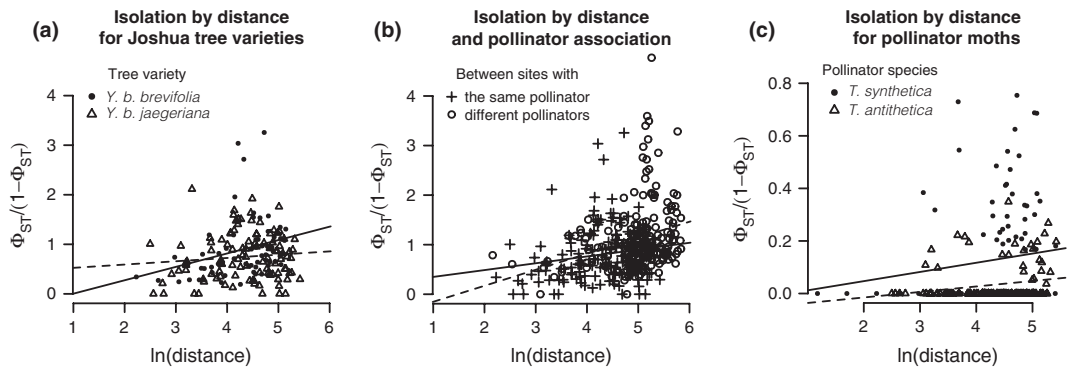


Fig. 3 Relationship between geographical and genetic distance for Joshua tree and its pollinators. Geographical vs. genetic distance scatter plot and regression line for (a) *Yucca brevifolia* var. *brevifolia* (dark circles, solid line) and *Y. b. jaegeriana* (open triangles, dashed line). (b) Geographical vs. genetic distances for Joshua tree sampling sites pollinated by the same moth species (crosses, solid line) and different moth species (open circles, dashed line). (c) Geographical vs. genetic distance scatter plot and regression line for *Tegeticula syntheticca* (dark circles, solid line) and *T. antithetica* (open triangles, dashed line).

Table 4 Parameter estimates from *Migrate-n* analysis.

Migration into	θ	Mutation-scaled rate of migration from		
		Western sites	Contact zone	Eastern sites
Western*	18.798 (15.4–22.3) [†]	–	6.471 (0–22.7) [‡] 30.4	0 (fixed)
Contact	0.843 (15.4–2.5)	171.560 (144.0–197.3) 36.2	–	114.367 (78.7–147.3) 24.2
Eastern	6.517 (3.5–9.4)	0 (fixed)	25.111 (5.3–44.0) 40.9	–

*Regions correspond to population groupings in Table 1.

[†]Parameter estimates given as mean (95% CI).

[‡]First line, mutation-scaled migration rate; second, corresponding rate in migrants/generation.

Effects of gene flow on trait matching between Joshua trees and moths

In the range-wide data set, we found the correlation between our summary of Joshua tree genetics and pollinator genetics is 0.475, significantly greater than zero (95% CI 0.098–0.732; $P = 0.016$). However, within the two separate plant–pollinator associations, no such correlation was present: for the western sites, the estimated correlation between plant and pollinator genetics is -0.219 (95% CI -0.771 to 0.521 ; $P = 0.571$); and for eastern sites, it is -0.150 (95% CI -0.648 to 0.437 ; $P = 0.624$).

Our model comparison approach identified substantially different associations among Joshua tree style length, moth ovipositor length, Joshua tree gene flow and pollinator gene flow at the different spatial scales we consider. In the range-wide data set, the best-fitting model predicted Joshua tree style length with only pollinator species identity (Table 5). The next

best-fitting model predicts style length using ovipositor length and moth genetics (Table 5; $\Delta\text{AICc} = 2.380$). The best-fit model identifies a strong positive effect of association with *T. syntheticca* on Joshua tree style length, which explains 85% of variation in style length.

When we compared the same models fitted only to the data from western populations pollinated by *T. syntheticca*, we found that the best-fitting model was the one in which moth genetics predicted Joshua tree style length (Table 5). However, this model did not explain more variation in style length than expected by chance ($F_{1,6} = 2.09$, $P = 0.198$), nor did any of the other candidate models (Table 5).

In contrast, for the eastern populations pollinated by *T. antithetica*, we found that the model predicting Joshua tree style length with Joshua tree genetics was the best-fit to style length (Table 5); and the model predicting style length with ovipositor length had a higher AICc score, but was not significantly worse-fit to the data (Table 5; $\Delta\text{AICc} = 1.859$). However, neither model

Table 5 Comparison of alternative models predicting Joshua tree style length.

Model: Style length predicted by	Range-wide ($n = 16$)			Western sites ($n = 8$)			Eastern sites ($n = 8$)		
	F	$P(F)$	ΔAICc^*	F	$P(F)$	ΔAICc	F	$P(F)$	ΔAICc
Ovipositor length	61.49	1.727×10^{-6}	4.387	0.14	0.722	2.208	1.47	0.270	1.859
Tree genetics	7.72	0.015	24.324	0.20	0.669	2.126	3.43	0.113	0.000
Moth genetics	58.69	2.259×10^{-6}	4.992	2.09	0.198	0.000	0.03	0.863	3.577
Moth species	85.31	2.476×10^{-7}	0.000						
Ovip. + tree gen. + ovip. \times tree gen.	18.5	8.518×10^{-5}	11.709	0.20	0.891	29.270	15.76	0.011	11.209
Ovip. + tree gen.	28.71	1.701×10^{-5}	7.951	0.33	0.732	10.724	1.68	0.277	8.849
Ovip. + moth gen. + ovip. \times moth gen.	27.37	1.191×10^{-5}	6.396	1.43	0.359	24.567	1.44	0.357	25.773
Ovip. + moth gen.	43.37	1.770×10^{-6}	2.380	0.87	0.473	9.328	0.67	0.554	11.062
Ovip. + moth sp. + ovip. \times moth sp.	26.67	1.359×10^{-5}	6.752						
Ovip. + moth sp.	39.85	2.849×10^{-6}	3.552						
Tree gen. + moth gen. + tree gen. \times moth gen.	17.63	1.077×10^{-4}	12.345	0.81	0.551	26.598	1.89	0.273	24.564
Tree gen. + moth gen.	28.41	1.798×10^{-5}	8.087	1.06	0.414	8.905	1.51	0.307	9.175

*All ΔAICc scores within 2 units of the best score are given in bold.

explained more variation in style length than expected by chance (respectively $F_{1,6} = 3.43$, $P = 0.113$; and $F_{1,6} = 1.47$, $P = 0.270$). The only candidate model that explained more variation in style length than expected by chance was the one incorporating ovipositor length, tree genetics and an interaction between the two (Table 5; $F_{3,4} = 15.76$; $P = 0.011$), but its AICc score was significantly worse than that of the best-fitting model, suggesting that the low P -value may be due to overfitting (Table 5; $\Delta\text{AICc} = 11.209$).

Discussion

Our analysis of neutral genetic variation across the range of Joshua tree demonstrates that pollinator-mediated reproductive isolation between the two morphologically distinct varieties of Joshua tree is incomplete at best. However, we do find evidence that pollinator specificity may have contributed to Joshua tree's divergence into two morphologically distinct varieties by restricting gene flow between the two varieties, and that patterns of asymmetric hybridization and introgression created by differential pollinator specificity in the Tikaboo Valley contact zone are mirrored at a broader geographical scale. Our work shows that the previously documented pattern of broad-scale trait matching between Joshua tree and its pollinators persists in the face of considerable recent or ongoing gene flow, which is consistent with the possibility that pollinator-mediated selection maintains the phenotypic differences between the two Joshua tree varieties. Within the two varieties of Joshua tree, *Y. b. brevifolia* and *Y. b. jaegeriana*, we find evidence suggesting that gene flow among Joshua tree populations may modulate phenotype matching in the eastern plant-pollinator association; in the west, we find that pollinator population structure may better predict Joshua tree phenotypes than either ovipositor length or tree population genetics.

Population genetic structuring by pollinator association

Our data demonstrate detectable, but by no means strong, genetic structuring between the two Joshua tree varieties based on their association with different pollinator species (Table 2, Figs 2 and 3b), and a larger proportion of genetic variation is attributable to the two tree varieties than is partitioned among sampling sites within each variety (Table 2). Bayesian clustering finds many ambiguously assigned individuals outside Tikaboo Valley, where the two Joshua tree varieties and their pollinators come into close contact, and hybridization is probably ongoing (Fig. 2). These patterns raise the possibility that the differentiation detected by our AMOVA is, in fact, the result of isolation by distance across the range of Joshua tree, which can lead to significant differentiation in AMOVA and the appearance of clustering in *Structure* (Meirns, 2012). However, when we compared the IBD relationships among pairs of sites with the same pollinator and pairs with different pollinator species, we found that isolation increases more rapidly with geographical distance between Joshua tree sites with different pollinators (Fig. 3b); this suggests that what differentiation we observe is not purely due to spatial isolation.

These results are consistent with evidence of hybridization between the two varieties of Joshua tree, which has probably occurred since the last glacial maximum (Smith *et al.*, 2008, 2011), and is likely ongoing (Smith *et al.*, 2009; Starr *et al.*, 2013). On the basis of their observed morphological differences and the discovery that they are pollinated by different species of yucca moths, taxonomists have proposed that the two varieties of Joshua tree be elevated to separate species (McKelvey, 1938; Lenz, 2007). Our data clearly show that reproductive isolation between *Yucca brevifolia* var. *brevifolia* and *Y. b.* var. *jaegeriana*, while detectable, is far from complete. Whether the two varieties of Joshua

tree are 'good' separate species in the light of this result is heavily dependent on the species concept we use to make that judgment (Hey, 2001, 2006; Coyne & Orr, 2004), and determining their taxonomic status is not a primary interest of this study.

Based on these results, it is not surprising that coalescent estimation of migration rates among the three major regions of Joshua tree's range identified by the *Structure* analysis strongly supports a high rate of gene flow into the Tikaboo Valley contact zone from 'pure' populations to the east and west, equivalent to rates of migration that should be more than sufficient to prevent neutral differentiation (Table 4; Wright, 1940). Relative to mutation, immigration from populations solely occupied by *Y. b. brevifolia* or solely by *Y. b. jaegeriana* is a substantial source of genetic variation for the Tikaboo Valley population (Table 4, Fig. S1). In contrast, Tikaboo Valley contributes relatively little variation to the pure populations, which makes sense in light of dramatic differences in the effective population sizes of the three regions (Table 4). Moreover, whereas the estimated contributions of genetic variation from the contact zone into *Y. b. jaegeriana* populations is low but nonzero, the 95% CI of our posterior estimate for gene flow into *Y. b. brevifolia* populations from Tikaboo Valley overlaps with zero. This mirrors the results of a recent fine-grained study of hybridization rates between the two Joshua tree varieties within Tikaboo Valley, which found that gene flow from hybrid trees to 'pure' individuals of *Y. b. jaegeriana* is greater than to individuals of *Y. b. brevifolia*, probably as a consequence of differences in host fidelity between the two pollinator species (Starr *et al.*, 2013).

We see much clearer evidence of isolation in our analysis of microsatellite variation in *Tegeticula synthetica* and *T. antithetica*, which are noninterbreeding species (Table 3; Smith *et al.*, 2008), and which display little to no isolation by distance within their respective ranges (Fig. 3c). Within the range of each individual pollinator species, genetic differentiation among pollinator sampling sites is not significantly better correlated with genetic differentiation among Joshua tree sampling sites than expected by chance. This result may be due to lack of power in our data set, or because variation in pollinator nuclear microsatellite loci may more strongly reflect movement of male moths, which do not carry pollen. Another yucca-associated Prodoxid, the nonpollinating yucca moth *Prodoxus coloradensis*, shows population genetic evidence of male-biased dispersal (Drummond *et al.*, 2009b). If dispersal is similarly male biased in Joshua tree's pollinators, population structure at nuclear markers may be only weakly related to pollen transport.

Phenotype matching between Joshua tree and its pollinators

Our model comparison approach finds that, at the range-wide level, variation in Joshua tree style length

is best explained simply by the species identity of the moths associated with each collection site, which explains 85% of variation in style length. The models with the next-best AICc scores predicted style length with the additive combination of ovipositor length and moth genetics (Table 5; $\Delta\text{AICc} = 2.380$, adjusted $R^2 = 0.850$) or with the additive combination of ovipositor length and moth species identity (Table 5; $\Delta\text{AICc} = 3.552$, adjusted $R^2 = 0.838$). These results, and the lack of support for models incorporating interactions between moth species identity and ovipositor length, or Joshua tree genetics and ovipositor length, recapitulate previous findings that the phenotypic differentiation between *Y. b. var. brevifolia* and *Y. b. var. jaegeriana* is overwhelmingly best explained by pollinator species (Godsoe *et al.*, 2008, 2010). Moreover, the poor fit of models incorporating interactions between moth species identity and ovipositor length suggests that our data cannot reject the hypothesis that there is no difference in the plant-pollinator phenotype-matching relationship for the two sets of associates.

Within each plant-pollinator association, however, we found a more complicated picture. The best-fit model to explain style length in the western populations does so using pollinator population genetics, which – although the model is not able to explain more variation than expected by chance – could be consistent with pollinator-mediated gene flow modulating trait matching at these sites (Table 5). However, this is unlikely because, as noted above, there is no significant correlation between Joshua tree and pollinator population structure in the west, which is an expected outcome if pollinator-mediated gene flow shapes Joshua tree population genetic structure. The second-best-fitting models using the western-only data predict style length with tree genetic structure or moth ovipositor length, and their difference in AICc score from the best-fitting model is barely more than the conventional threshold for significance (Table 5; $\Delta\text{AICc} = 2.126$ and 2.208 respectively). We note that none of the candidate models fitted to the western style length data explained more variation than expected by chance (Table 5), which may indicate poor statistical power due to the small sample size created by subdividing the range-wide data. We suspect, therefore, that a larger data set would reveal that moth ovipositor length and Joshua tree genetics, or some interaction between the two, are also important predictors of style length in western populations – and that the effect of moth population structure on tree style length is mediated by moth ovipositor length.

In the eastern collection sites, we find that variation in style length is best explained by Joshua tree population genetics, although not significantly better than it is explained by pollinator ovipositor length (Table 5). Neither of these models explain more variation than expected by chance, but together, they are consistent with the hypotheses that gene flow and pollinator-

mediated selection determine the observed variation in style length in eastern Joshua tree populations. The model explaining style length with ovipositor length, Joshua tree genetics and an interaction between the two is, intriguingly, the only candidate model to explain more style-length variation in eastern sites than expected by chance, but its poor AICc score may mean that it suffers from overfitting. As in the western sites, then, we must suspect that our data for the eastern plant–pollinator association lack power to test this more complex hypothesis.

With the necessary caveats about statistical power in mind, what could explain the different patterns we see in each variety of Joshua tree? Differential degrees of gene flow within each variety is one possibility; if gene flow among sites is weaker in *Y. b. jaegeriana* than in *Y. b. brevifolia*, then coevolutionary selection would also be stronger, relative to gene flow, in *Y. b. jaegeriana*. However, our analysis of pollinator population genetics suggests that both pollinators disperse widely: in both moth species, a very small proportion of genetic variation is partitioned among sampling sites (Table 3), and neither pollinator shows much evidence of isolation by distance (Fig. 3c). Alternatively, it may be that coevolutionary selection is actually stronger in *Y. b. jaegeriana* than in *Y. b. brevifolia*. We also note that population expansion since the last glacial maximum may mean that neutral genetic diversity, for both Joshua tree and its pollinators, is not in migration-drift equilibrium (Smith *et al.*, 2011). Determining the relative importance of local coevolutionary selection and gene flow will be an important question for future study in this system, particularly at the Tikaboo Valley contact zone. However, in the absence of direct measurements of the fitness consequences of plant–pollinator mismatch for Joshua trees, our data set allows only speculation as to which forces most strongly shape the phenotypic and genetic diversity we observe (Gomulkiewicz *et al.*, 2007; Nuismer *et al.*, 2010).

Pollination interactions and speciation of flowering plants

Although the association between specialized animal pollination and speciation in flowering plants has been well established over decades of empirical and theoretical study (Grant, 1949; Kiester *et al.*, 1984; Hodges & Arnold, 1995; Sargent, 2004), we still have relatively little data to determine how often pollination interactions directly cause speciation (Armbruster & Muchhala, 2009). Comparative theoretical work suggests that relative to antagonistic species interactions such as parasitism and competition, the coevolution of plants and their pollinators is unlikely to generate greater phenotypic diversity within species, and may even act to reduce diversity (Kopp & Gavrillets, 2006; Yoder & Nuismer, 2010). The data and analyses we present here are correlative, and

cannot conclusively test the hypothesis that Joshua tree's divergence was caused by the speciation of its pollinators (Gomulkiewicz *et al.*, 2007; Nuismer *et al.*, 2010). However, our results do demonstrate that the interaction between Joshua tree and its pollinators probably restricts the rate of pollen transfer between the two varieties of Joshua tree (Table 2; Fig. 2, Fig. 3c), and may create ongoing divergent natural selection on the floral morphology of those two varieties (Table 5).

This suggests a possible way by which specialized pollination interactions can contribute to increased rates of speciation without directly creating reproductive isolation. If specialized pollination systems tend to generate coevolutionary selection that restricts diversification within species (Yoder & Nuismer, 2010), then plant–pollinator interactions may accelerate divergence in populations of plants and pollinators that have first been separated by some noncoevolutionary process, resulting in reproductive isolation on secondary contact (Kiester *et al.*, 1984). Because pollination is directly linked to mating in plants, pollinators may act as a sort of 'magic trait' by proxy – their speciation in response to ecological selection or a vicariance event has the side effect of reducing gene flow between populations of the plants they service (Gavrilets, 2003; Coyne & Orr, 2004). The opportunity for a species to undergo adaptive divergence increases as the number of traits under divergent selection increases (Nosil *et al.*, 2009), and to the extent that they are sensitive to environmental factors independent of those that directly affect their host plants, pollinators indirectly expose their hosts to new sources of isolation and divergent selection.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Mutation-scaled population size and asymmetric rates of migration in Joshua tree.

Table S1 Observed and expected heterozygosity for Joshua tree microsatellite loci at all collection sites.

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