

Host specificity and reproductive success of yucca moths (*Tegeticula* spp. Lepidoptera: Prodoxidae) mirror patterns of gene flow between host plant varieties of the Joshua tree (*Yucca brevifolia*: Agavaceae)

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Abstract

Coevolution between flowering plants and their pollinators is thought to have generated much of the diversity of life on Earth, but the population processes that may have produced these macroevolutionary patterns remain unclear. Mathematical models of coevolution in obligate pollination mutualisms suggest that phenotype matching between plants and their pollinators can generate reproductive isolation. Here, we test this hypothesis using a natural experiment that examines the role of natural selection on phenotype matching between yuccas and yucca moths (*Tegeticula* spp.) in mediating reproductive isolation between two varieties of Joshua tree (*Yucca brevifolia* var. *brevifolia* and *Y. brevifolia* var. *jaegeriana*). Using passive monitoring techniques, DNA barcoding, microsatellite DNA genotyping, and sibship reconstruction, we track host specificity and the fitness consequences of host choice in a zone of sympatry. We show that the two moth species differ in their degree of host specificity and that oviposition on a foreign host plant results in the production of fewer offspring. This difference in host specificity between the two moth species mirrors patterns of chloroplast introgression from west to east between host varieties, suggesting that natural selection acting on pollinator phenotypes mediates gene flow and reproductive isolation between Joshua-tree varieties.

Keywords: coevolution, gene flow, host specificity, microsatellite markers, mutualisms, pollination, sibship reconstruction, speciation

Received 23 April 2009; revision received 1 July 2009; accepted 9 July 2009

Introduction

Macroevolutionary patterns suggest that coevolution between plants and insects has profoundly shaped the evolution of life on Earth (Ehrlich & Raven 1964; Thompson 1994, 2005). For example, sister-group comparisons indicate that phytophagy is associated with increased diversity in many insect groups (Mitter *et al.* 1988; Farrell 1998), and reliance on specialized pollinators appears to have promoted the diversification of many angiosperm lineages (Grant 1949; Sargent 2004).

However, the specific mechanisms underlying this correlation remain unclear (Armbruster & Muchhala 2009).

Obligate pollination mutualisms, such as the interactions between yuccas and yucca moths, have been widely recognized as model systems for studying coevolution and its role in promoting speciation (Pellmyr 2003). Within these systems, the plants are typically pollinated by a single species that reproduces solely by laying its eggs in flowers (Janzen 1979; Holland & Fleming 1999; Weiblen 2002; Kato *et al.* 2003). Furthermore, these systems commonly display matching between plant and pollinator phenotypes. For example, among figs and fig wasps, there is a significant correlation between the length of the female fig wasp's

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ovipositor, and the length of the floral style (Weiblen 2004). Similarly, within yuccas and yucca moths, there is a significant correlation across species between moth ovipositor length and the thickness of the ovary wall at the point of oviposition (O. Pellmyr, unpublished data).

Mathematical models show that, in principle, selection for matching of plant and pollinator features in local demes could indirectly promote reproductive isolation between populations, and thereby drive rapid speciation in these systems (Kiestler *et al.* 1984). Empirical tests of this hypothesis are few, in part because the biology of these systems generally makes experimentation to quantify natural selection and reproductive isolation difficult. The plants typically require many years to reach reproductive maturity, and the pollinators' strict reliance on mature hosts for reproduction makes it difficult to maintain them as laboratory stocks that could be used for selection experiments.

One such pollination mutualism, the interaction between the Joshua tree [*Yucca brevifolia* Engelm. (Agavaceae)] and its pollinating moths [*Tegeticula antithetica* Pellmyr and *T. synthetica* Riley (Lepidoptera: Prodoxidae)], offers significant promise for testing the role of selection on phenotype matching in promoting speciation. Joshua trees, like all yuccas, are pollinated exclusively by yucca moths (Pellmyr 2003). Female yucca moths lay their eggs in Joshua tree flowers by inserting a flattened, blade-like ovipositor through the top of the wall of the floral pistil and down the stylar canal (Trelease 1893). Following oviposition, to ensure that the flower will develop and produce seeds for the offspring to eat, the moths deliberately pollinate the flower, placing pollen collected previously directly onto the floral stigma using uniquely-derived, tentacle-like mouthparts (Pellmyr 2003).

Across its range, Joshua tree is associated with two distinct, parapatrically-distributed, sister-species of moths, *T. synthetica* occurring in the western portion of its range, and *T. antithetica* in the eastern portion (Pellmyr & Segraves 2003). These moths differ significantly in body size (Fig. 1A), and in the size of the female ovipositor, with the ovipositor of the western moth (*T. synthetica*) being about 70% larger than that of the eastern species (*T. antithetica*) (Pellmyr & Segraves 2003). Likewise, morphological studies have shown that Joshua trees pollinated by the two moth species differ significantly in both vegetative and floral features (Rowlands 1978; Lenz 2007; Godsoe *et al.* 2008), with the greatest difference found in the length of the stylar canal – the path through which the female yucca moth inserts her ovipositor during oviposition (Fig. 1B) (Godsoe *et al.* 2008).

The remarkable match between the length of the female yucca moth's ovipositor and the length of the

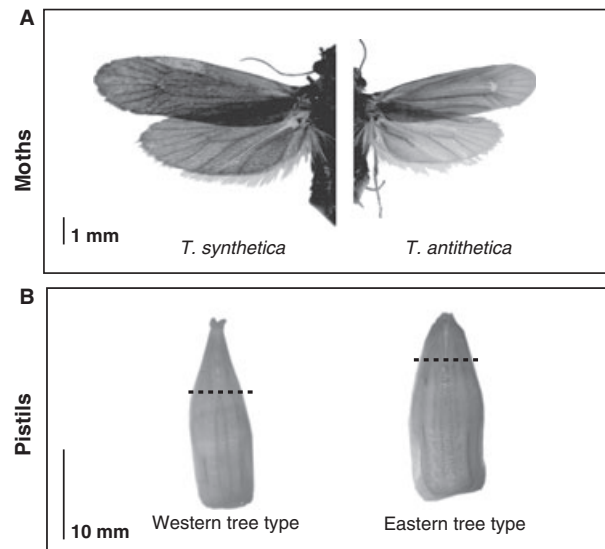


Fig. 1 Phenotypes of Joshua tree flowers and their pollinators in Tikaboo Valley, Nevada. A: Relative body size of the western moth *Tegeticula synthetica* and the eastern moth *T. antithetica*, B: Cross-sectioned flowers of trees pollinated by each from the Tikaboo Valley contact zone. The floral stylar canal extends from the top of the column of ovules (dotted line) to the tip of the stigma.

floral stylar canal likely reflects important functional constraints acting on both the plant and the pollinator. The moth's ovipositor must be long enough to reach the ovules, but not so long as to inflict significant injury to the developing flower. Work examining *Y. filamentosa* has shown that the plants may prevent over-exploitation of the seeds by selectively aborting flowers that have received too much oviposition-related damage, killing both the seeds and developing moth larvae (Pellmyr & Huth 1994; Marr & Pellmyr 2003). Similarly, it has been argued that lengthening of the pistil in *Y. baccata* prevents pollinators from reaching and successfully ovipositing into viable ovules (Bao & Addicott 1998). The conflict between the plant's interests (minimizing the number of seeds lost to feeding by the moth's larvae) and the moth's interests (producing as many larvae as possible), sets up a coevolutionary tug-of-war between plant and pollinator that has shaped the evolutionary history of each (Pellmyr 2003).

Based on their morphological differences, and on the differences in pollinator affinities, taxonomists have recommended the recognition of eastern and western Joshua tree varieties as distinct species (McKelvey 1938; Lenz 2007), but the validity of this designation is as yet uncertain. While the pollinator species associated with the two Joshua tree varieties are clearly reproductively isolated, with no evidence for gene flow or hybridization between species, chloroplast DNA (cpDNA)

sequence data indicate appreciable levels of ongoing gene flow between the two tree varieties (Smith *et al.* 2008a). Interestingly, these data also suggest a striking asymmetry in the degree of isolation between the two varieties. Whereas per-generation rates of gene flow from populations of eastern trees into populations of western trees were not significantly different from zero, there was significant evidence for ongoing chloroplast gene flow from west to east (Smith *et al.* 2008a).

What the chloroplast data indicate about reproductive isolation between tree varieties is unclear however. The chloroplast is maternally inherited in most flowering plants, including yuccas, passed through seeds but not through pollen, so these data do not reflect pollinator-mediated gene flow, at least not directly. The observed pattern of cpDNA introgression could be explained by either introgression of the cpDNA genome into eastern trees via epistasis and seed-mediated gene flow, or chloroplast capture (Rieseberg & Soltis 1991) via pollinator-mediated nuclear DNA (nucDNA) gene flow. The first explanation seems unlikely, as the geographic pattern of introgression would require seed dispersal across hundreds of kilometers, while empirical studies suggest that seeds typically do not travel more than 10–30 m from the maternal plant (Vander Wall *et al.* 2006). If the latter hypothesis were correct, however, then the marked asymmetries in the direction of gene flow would suggest a substantial difference in the host fidelity of the two pollinator species. That is, in order for the high levels of *chloroplast* introgression from west to east to be explained by *nuclear* gene flow, there must be significant pollinator mediated gene flow from *east to*

west. Thus, we would predict that the eastern moth (*T. antithetica*) must visit and successfully pollinate western trees much more often than western moth (*T. synthetica*) pollinates eastern trees (Fig. 2).

Although reciprocal transplant experiments are a common approach to quantifying host specificity and natural selection in host/parasite interactions, such an experiment in this system would be logistically quite challenging, as it would require excluding pollinators from experimental trees, experimentally introducing moths onto native and foreign hosts, and sufficient replication to overcome the high frequency with which trees fail to set fruit altogether. However, the existence of a zone of secondary contact presents a natural laboratory in which the reciprocal transplant experiment can be approximated using observational data. The two moth species, and the two Joshua tree varieties, co-occur in a narrow contact zone ~4 km wide in Tikaboo Valley, Nevada (Fig. 3B) (Rowlands 1978; Smith *et al.* 2008a). Here, the two pollinator-associated tree varieties occur side-by-side (Fig. 3A), along with both of the two yucca moth species. The area of sympatry presents a unique opportunity to track the two moth species' natural rates of visitation and oviposition success on different hosts, offering a more practical alternative to a manipulative reciprocal transplant experiment.

Here, we report data from a natural experiment that examines host specificity and the fitness consequences of oviposition on native and foreign hosts. We used glue traps to measure the rate at which the two moth species visit the two tree varieties in this contact zone, and DNA barcoding of larvae to measure the rate at

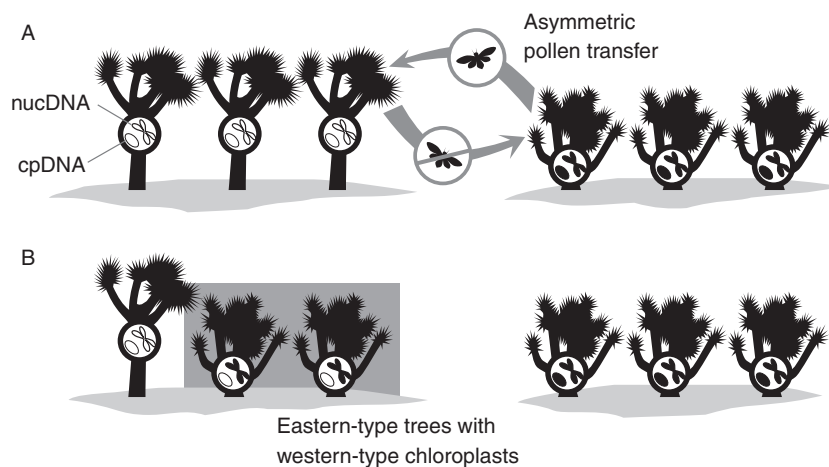


Fig. 2 Differences in host specificity may have caused chloroplast introgression between tree types. In the past (A), eastern and western trees may have been genetically distinct in both nuclear and chloroplast genomes. Pollination of western trees by eastern (*T. antithetica*) moths may have introduced eastern genes into populations of western trees. Because the chloroplast is not transmitted in pollen, differences in pollinator specificity would have had no effect on the distribution of chloroplast genomes. Over time, (B) the constant input of eastern genetic material could have converted formerly western trees to an eastern phenotype, producing morphologically eastern trees that retain western chloroplasts.

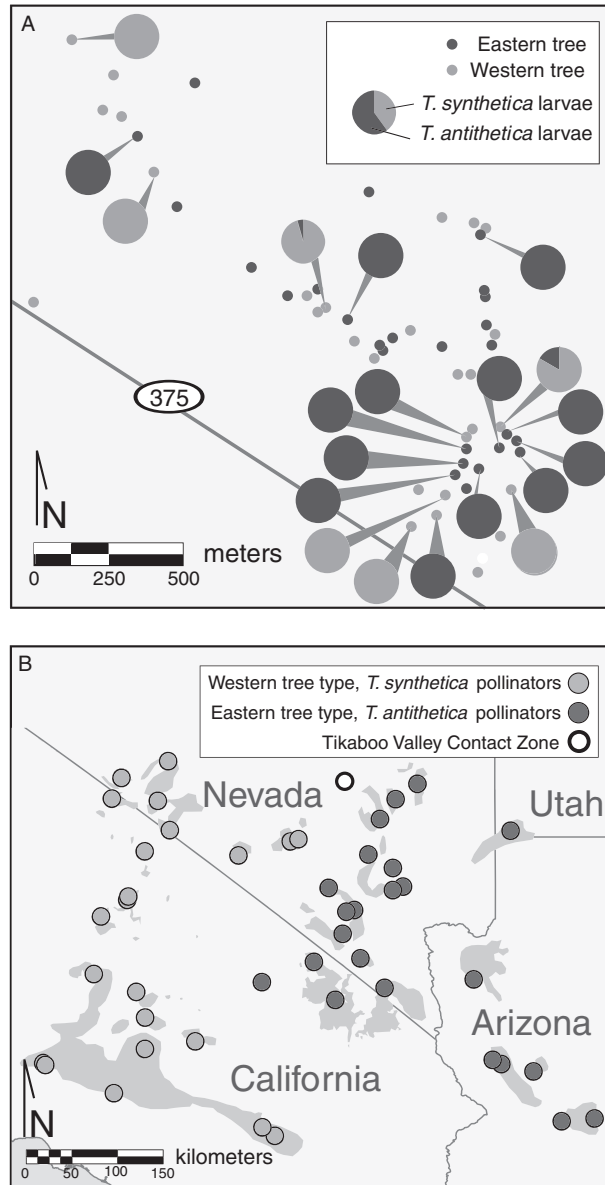


Fig. 3 Location of the study site and distribution of study trees. A: Map of the Tikaboo Valley contact zone. Small circles show location of individual study trees. Coloration of trees shows tree varietal status, determined by logistic regression of morphological data. Pie chart call-outs show frequency of western (blue) and eastern (green) larvae reared from fruit on each tree. B: Geographic range of Joshua tree and its pollinators. The Tikaboo Valley contact zone is shown as a hollow circle near the northern edge of the range.

which the two moth species successfully pollinate and oviposit onto the two tree varieties. Last, we genotyped larvae using microsatellite DNA markers, allowing us to estimate maternal sibship groups and the average clutch size (hence, reproductive success per oviposition effort) of moths ovipositing on native and foreign hosts. The results show that the two moth species differ significantly in their degree of host specificity, and that oviposition on foreign hosts imposes a significant cost in terms of reduced clutch size. These findings in turn suggest that differences in the ovipositor morphology of

the two moth species, and resulting differences in pollination and oviposition success on each host, may explain the asymmetric rates of gene flow between tree varieties observed previously and provide a mechanism of reproductive isolation between tree varieties.

Methods

In late March of 2007, Joshua trees in the Tikaboo Valley contact zone were surveyed along an ~2 km belt transect. Forty-two trees were identified as either

'eastern' or 'western' varieties based on gross morphology and the height of first branching; trees with height to first branching > 1 m were designated as western (*Y. brevifolia* var *brevifolia*), and those with height to first branching < 1 m were designated as eastern (*Y. brevifolia* var *jaegeriana*) [cf. (Godsoe *et al.* 2008)]. Two morphologically intermediate trees that could not be unambiguously assigned to one variety or the other were omitted from this study.

Following the appearance of the first buds on the trees, but before any buds had opened, a sticky card glue trap was attached to one bud on each tree using a plastic tie-down secured around the peduncle. After buds had opened, flowers were collected from unmonitored inflorescences on each study tree to confirm tree varietal assignments using floral measurements. The cards were left in place for 24 days, until after peak pollinator emergence. At this time, cards were removed, transported to the laboratory, and stored at -80°C, pending moth morphotyping.

The lengths of the forewings of moths caught on the sticky cards were measured using an optical micrometer under an Olympus SZX-16 stereo dissecting microscope. Based on previous anatomical studies of these insects (Pellmyr & Segraves 2003) and on the frequency distribution of moth wing lengths observed on the sticky cards, moths with forewings of 6.8 mm or less in length were designated as *T. antithetica*, those of 7.0 mm or greater in length were designated as *T. synthetica*.

In May 2007, fruit were collected from monitored trees. Because only a small fraction of study trees set fruit, collections were supplemented with fruit from an additional 20 trees sampled along a second transect, which were typed to varietal status based on gross morphology. Fruit were transported to the laboratory and separated into individual plastic bins, labeled by tree of origin. Fruit were kept moist by spraying them with water daily, and were monitored twice daily for larval emergence. Emerging larvae were collected in individual 1.5 mL microcentrifuge tubes, labeled as to the tree and fruit of origin, and stored at -80°C, pending genotyping. After larval emergence had ceased, each fruit was dissected to remove any remaining larvae. A small number of larvae (13) emerged during shipping from the field to the laboratory, and before fruits had been separated into individual containers. Because they could be associated with a particular tree, but not a specific fruit, these individuals were excluded from per-fruit estimates of larval emergence (below), but were included in measures of the rates of larval emergence per tree type.

Reliable morphological characters for distinguishing *T. synthetica* larvae from *T. antithetica* larvae have not

been identified, so larvae were assigned to species using DNA barcoding and microsatellite DNA genotyping. Genomic DNA was extracted from 713 individuals of *T. antithetica* and *T. synthetica*: (i) 222 moths and larvae collected across the range of *Y. brevifolia* (*T. antithetica* adults, $n = 58$; *T. synthetica* adults, $n = 67$; unidentified larvae, $n = 97$); (ii) and 491 moths and larvae from the Tikaboo Valley contact zone (*T. antithetica* adults, $n = 2$; *T. synthetica* adults, $n = 8$; unidentified larvae, $n = 481$).

For the 481 unidentified moth larvae from the contact zone, the 5' end of the mitochondrial gene Cytochrome Oxidase One (COI) was amplified by PCR and sequenced in one direction using ABI Big Dye® v. 3.1 dye terminators (Applied Biosystems), analyzed in an ABI 3730 capillary sequencer. Detailed PCR and sequencing protocols are described in Smith *et al.* (2008a). Multilocus microsatellite DNA genotypes were obtained from nine loci following the procedures described in Drummond *et al.* (2009), including replicate genotyping of samples to ascertain reproducibility and error rates. Microsatellite DNA electrophoresis was performed on an ABI 3130 capillary instrument using 1 µL of multiplex PCR product in 10 µL HiDi formamide and 0.15 µL Genescan 500 LIZ size standard (Applied Biosystems), and scored in GeneMapper 4.0 (Applied Biosystems).

Resulting microsatellite DNA genotypes and mtDNA sequences were used to assign individuals to either *T. antithetica* or *T. synthetica*. mtDNA sequences were edited in CodonCode Aligner version 2.04, using automated end clipping and SNP detection; for unique mutations the original chromatograms were used to check for base-calling errors. Sequences were output as PHYLIP files and aligned to outgroup sequences (*Lampronia rubiella*, *Prodoxus sordidus*, *Parategeticula elephantipella*, *T. maculata*, and *T. yuccasella*), as well as previously sequenced samples from adult *T. synthetica* (4) and *T. antithetica* (2) using ClustalW (Thompson *et al.* 1994). Aligned mtDNA sequences were analyzed in PAUP* v. 3.0b10 (Swofford 2002) to infer phylogenetic relationships among mtDNA haplotypes using neighbor joining and an heuristic search implemented under a parsimony criterion (see Results, below). Because the two moth species were strongly supported as reciprocally monophyletic in previous phylogenetic studies (Smith *et al.* 2008a), larval mitotypes were assigned to species based on whether they fell into the clade containing known adult individuals of *T. synthetica* or *T. antithetica*.

Microsatellite DNA genotypes were analyzed using Bayesian clustering in structure 2.3.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007) using the complete set of 713 individual moths described earlier, including known adult individuals and larvae sampled from both

Tikaboo Valley and across the range of each moth species. Bayesian clustering was implemented under a model with no a priori designation of populations, correlated allele frequencies, independent estimates of α for each cluster, and locus-specific null alleles based on the presence of putative null homozygotes. Three replicate Bayesian Markov chain Monte Carlo (MCMC) runs were performed for $K = 1-8$, allowing 100 000 burn-in iterations and 500 000 posterior iterations for $K = 1-5$ and 200 000 burn-in iterations and 1 000 000 posterior iterations for $K = 6-8$. We inspected trace plots for all parameters to insure adequate burn-in as well as stationarity and convergence of independent MCMC runs. To assess the uppermost level of hierarchically nested structure, we evaluated plots of $\ln(P|D)$ and ΔK (Evanno *et al.* 2005) for increasing values of K .

To determine whether moths pay a fitness cost for ovipositing on the non-native host, we next used microsatellite DNA to infer family relationships of larvae within and between fruits. The average clutch size (the number of offspring each female moth successfully rears from a given fruit) represents the reproductive success per moth per oviposition event. To determine clutch sizes, pairwise sibship or relationship classes for Tikaboo Valley larvae from each species were assigned using the maximum likelihood (ML) simulated annealed algorithm implemented in COLONY 2.0 (Wang 2004). In addition, maternal sibships were excluded for individuals with different mtDNA haplotypes. These data together were used to assign individual larvae to putative matriline. Based on the assignments of larvae to matriline, we counted the number of larvae produced by each female on each fruit – that is, the number of larvae produced per successful oviposition.

Because inference of individual relationships depends on accurate estimates of background allele frequencies, the sample of Tikaboo Valley larvae was split into *T. antithetica* and *T. synthetica* on the basis of concordant results for species identification from both microsatellite DNA clustering and the mtDNA phylogenetic analysis. Summary statistics for each taxon and microsatellite DNA locus were calculated using Genepop 4.0 (Rousset 2008), MSA 4.05 (Dieringer & Schlötterer 2003), FreeNA (Chapuis & Estoup 2006), and Pedant 1.0 (Johnson & Haydon 2007a,b). Three replicate COLONY analyses were conducted using the ‘medium’ run length option, including locus-specific estimates of error rates due to allelic dropout and/or allelic mismatches. To evaluate the informativeness of the microsatellite DNA data for sibship assignment, multilocus parental exclusion probabilities and power to discriminate between three relevant relationship classes (full-sib vs. unrelated, full-sib vs. half sib, half-sib vs. unrelated) were estimated using

KinInfor 1.0 (Wang 2006) based on the observed frequencies of microsatellite DNA alleles for each species at Tikaboo Valley.

We determined if observed frequencies of moth visitation and larval emergence rates could occur in the absence of specialization using Chi squared tests. For these tests, the null expectation is that that emergence and visitation rates are independent of host type. Likewise, we determined whether the overall rates of larval emergence were independent of adult visitation rates using a chi-squared test. Next, to examine whether each particular moth species produced significantly more larvae on its native host, we used a binomial test. As in the overall comparisons using Chi-squared tests, this test determined whether the observed number of larvae was significantly different from either the null hypothesis of equal success on each tree type, or success in proportion to observed adult visitation on each tree type. Finally, the average numbers of larvae per sibship, per fruit (i.e. the average clutch size and hence the moths’ reproductive success per oviposition event) were compared between moth species, and within moth species between tree types, using paired t-tests assuming unequal variance.

Results

Of the 42 sticky cards originally placed, nine were removed by browsing animals during the experiment, leaving 16 cards from western trees and 17 cards from eastern trees. Of the moths caught on these cards, 58 could be identified to species based on forewing length. 32 of these were identified as *T. antithetica*, and 26 as *T. synthetica*. (Table 1A). A small, but uncounted, number were too badly damaged by the glue on the sticky traps

Table 1 Moth host specificity as measured by moths caught in sticky traps on each host type, and larvae reared from fruit

A. Moth visitation rates		
Tree type	Moth species (forewing length)	
	<i>T. antithetica</i>	<i>T. synthetica</i>
Eastern	10	3
Western	22	23

B. Larval emergence rates		
Tree type	Larval mitotype	
	<i>T. antithetica</i>	<i>T. synthetica</i>
Eastern	378	0
Western	12	91

to allow reliable designation to species, and were excluded from subsequent analyses.

Phylogenetic analysis of the larval mtDNA haplotypes using neighbor joining revealed two reciprocally-monophyletic clades containing *T. antithetica* and *T. synthetica*, respectively, subtended by a paraphyletic grade of four sequences (Supplemental Online Materials Fig. S1). The individuals in this grade were those for which only relative short (~250 bp) sequence reads were obtained (most sequences were > 650 bp in length). All four of these were classified unambiguously as *T. synthetica* in the Structure analysis (below). To further explore the placement of these four sequences, a second phylogenetic analysis was completed using parsimony, including a small ($n = 20$), random subsample of ingroup taxa and five outgroups. The results of this analysis (not shown) revealed that under a parsimony criterion all four of the sequences that formed the basal grade in the neighbor-joining tree could be unambiguously placed within the *T. synthetica* clade, with moderate bootstrap support (72%) for the monophyly of *T. synthetica*. We therefore scored these four samples as *T. synthetica* for purposes of estimating larval emergence rates.

Bayesian clustering at $K = 2$ divided the sample into two distinct genetic groups (see Supplemental Online Materials Fig. S2), with no evidence for significant admixture from 95% credibility intervals for coancestry. All adults identified to species by morphology were placed in the correct cluster (see Supplemental Materials Fig. S2). Apart from the four individuals forming the basal grade in the neighbor-joining analysis (above), all assignments based on microsatellite DNA genotypes agreed with the placements based on phylogenetic analysis of mtDNA sequence data. Although $\ln(P|D)$ values continued to increase up to $K = 8$, ΔK values showed a sharp peak at $K = 2$ (see Supplemental Online Materials Fig. S3), strongly suggesting that the uppermost level of genetic structure matches the division between *T. antithetica* and *T. synthetica*, with additional substructuring as a consequence of population subdivision and intensive sampling of family groups at Tikaboo Valley.

Four hundred and eighty one larvae emerged from collected fruits, of which 390 were identified as *T. antithetica*, and 91 were identified as *T. synthetica* (Table 1B). A total of 12 *T. antithetica* larvae emerged from western trees. No *T. synthetica* larvae were recovered from their non-native (eastern) trees. Adult visitation rates on the two tree types did not differ significantly from the null expectation ($\chi^2 = 3.21$, $P = 0.068$, d.f. = 1); although eastern trees received very few visits by western (*T. synthetica*) moths, western trees received almost exactly equal numbers of the two

moth species (22 *T. antithetica* vs. 23 *T. synthetica*). However, larval emergence differed significantly from the null expectation of equal proportions ($\chi^2 = 411.89$, $P < 0.0001$, d.f. = 1), and from the adult visitation rates ($\chi^2 = 1284.61$, $P < 0.0001$, d.f. = 3). The binomial tests show that *T. synthetica* produced significantly fewer larvae on eastern trees than could be explained by chance ($P < 0.00001$) or than could be explained if larval emergence was proportional to adult visitation ($P = 0.000014$). Likewise, *T. antithetica* produced significantly fewer larvae on western trees than could be explained by chance ($P < 0.00001$) or than could be explained if larval emergence was proportional to adult visitation ($P < 0.00001$).

Maximum likelihood assignment of pair-wise relationship classes identified 57 matriline within *T. antithetica*, with an average of 6.85 ± 6.76 larvae per female. Within *T. synthetica*, 25 sibships were identified with an average of 3.64 ± 2.69 larvae per female. COLONY output for five typical matriline from each species, showing the distribution of larvae among fruits and among trees are presented in Tables 2 and 3 (*T. antithetica* and *T. synthetica*, respectively). Representative patterns of larval sibship among fruit and trees are shown as pair-wise plots in Fig. 4. The full pairwise plots for all genotyped individuals and full COLONY output can be found in the supplementary online material Table S1. Individual female *T. antithetica* matriline were often spread among multiple fruits within a tree and among multiple trees, whereas *T. synthetica* larvae from a given matriline typically all occurred on the same tree. Two matriline were spread across both eastern and western trees, but in both cases the larvae on different tree types were half-sibs. Results from KinInfo 1.0 (Wang 2006) suggest that the microsatellite DNA data were able to distinguish full sibs from unrelated individuals with 96.2% and 92.9% accuracy for *T. antithetica* and *T. synthetica*, respectively. However, the power to distinguish half sibs from unrelated individuals was much lower, 66% for *T. antithetica* and 58% for *T. synthetica*.

Average clutch sizes per female per fruit (i.e. the reproductive output per oviposition event) are summarized in Fig 5. The average clutch size for *T. antithetica* females ovipositing onto western trees (the non-native host) was significantly smaller than those ovipositing onto eastern trees (the native host) (3.51 vs. 1.71, $P = 0.0014$, two-tailed t-test). *Tegeticula antithetica* also showed larger clutch sizes when ovipositing onto its native (eastern) host than *T. synthetica* on its native (western) hosts (3.51 vs. 2.33, $p = 0.0072$, two-tailed t-test). Because *T. synthetica* larvae were never recovered from eastern trees, no data are available on the average clutch size for this species on its non-native host.

Table 2 Five typical matrilineal assignments for *T. antithetica* larvae as inferred using COLONY 2.0. Results for all 390 larvae, representing 57 matrilineal, are available as supplemental online material Table S2

Offspring ID	Father ID	Mother ID	Tree	Fruit
07_1303	*12	#13	E1	5
07_1304	*12	#13	E1	5
07_1306	*12	#13	E25	1
07_1343	*12	#13	E35	3
07_1279	*13	#14	E1	6
07_1280	*13	#14	E1	6
07_1281	*13	#14	E1	6
07_1286	*13	#14	E1	6
07_1287	*13	#14	E1	6
07_1389	*13	#14	E1	8
07_1390	*13	#14	E1	8
07_1391	*13	#14	E1	8
07_1392	*13	#14	E1	8
07_1345	*13	#14	E1	28
07_1346	*13	#14	E1	28
07_1388	*13	#14	E1	28
07_1549	*13	#14	E1	28
07_1333	*13	#14	E39	3
07_1305	*14	#15	E1	6
07_1250	*14	#15	E39	2
07_1251	*55	#15	E39	2
07_1252	*55	#15	E39	2
07_1289	*14	#15	E39	3
07_1402	*55	#15	E39	3
07_1393	*13	#16	E1	7
07_1256	*13	#16	E1	20
07_1348	*13	#16	E1	28
07_1394	*15	#17	E1	7
07_1621	*19	#17	E1	16
07_1038	*15	#17	E1	20
07_1039	*15	#17	E1	20
07_1073	*15	#17	E1	20
07_1074	*15	#17	E1	20
07_1148	*15	#17	E1	20

Table 3 Five typical matrilineal assignments for *T. synthetica* larvae as inferred using COLONY 2.0. Results for all 91 larvae, representing 25 matrilineal, are available as supplemental online material Table S3

Offspring ID	Father ID	Mother ID	Tree	Fruit
07_1891	*4	#3	W14	11
07_1395	*4	#3	W14	15
07_1396	*4	#3	W14	15
07_1397	*4	#3	W14	15
07_1589	*3	#4	W14	17
07_1590	*3	#4	W14	17
07_1144	*5	#5	W14	27
07_1898	*6	#6	W14	35
07_1580	*7	#7	W14	37
07_1587	*7	#7	W14	37
07_1588	*7	#7	W14	37

between host varieties – show that phenotype matching may indeed play an important role in mediating reproductive isolation.

Host specificity in yucca moths that pollinate Joshua tree is maintained in sympatry (Fig. 3), but the degree of host specificity differs between moth species. Although small sample sizes limit our statistical power, and although the Chi-squared test was marginally non-significant ($P = 0.068$), it seems that the western moth species, *T. synthetica*, visits eastern trees only rarely, but the eastern moth species, *T. antithetica*, visit eastern and western trees indiscriminately, and western trees appear to receive equal visitation by both species. This observation predicts higher rates of gene flow from eastern trees to western trees than vice-versa and is strikingly consistent with the patterns of chloroplast introgression from west to east described previously (Smith *et al.* 2008a).

Past work on Joshua trees has advanced the argument that phenotype matching between the length of the moth's ovipositor and the floral style provides a mechanism of reproductive isolation between tree types (Godsoe *et al.* 2008). Given the patterns of phenotype matching between moth and floral characters seen in this system, and given the indications of strong functional constraints on moth ovipositor morphology, it is not difficult to imagine that attempted oviposition on the non-preferred host could have different fitness consequences for the two moth species. For example, as excessive oviposition-related damage is known to prompt floral abscission in other yuccas, it seems reasonable to expect that the larger, western moth (*T. synthetica*) may be likely to induce abscission when ovipositing onto eastern trees, killing any prospective hybrid seeds. In contrast, if the smaller, eastern moth (*T. antithetica*) were to attempt oviposition onto western trees, the moth might produce fewer larvae as a

Discussion

The idea that pollinator specialization may promote speciation in host plants represents a long-standing hypothesis in evolutionary ecology (Darwin 1876; Grant 1949; Stebbins 1970; Hodges & Arnold 1995; Sargent 2004; Good-Avila *et al.* 2006; Smith *et al.* 2008b), but the specific mechanisms by which pollinators might promote speciation have remained obscure (Armbruster & Muchhala 2009). It has been suggested that natural selection for matching of plant and pollinator phenotypes could indirectly favor reproductive isolation between plant populations (Kiestler *et al.* 1984), but empirical support for this model has been sparse. Our results – showing that natural selection acting on pollinator phenotypes match differences in gene flow

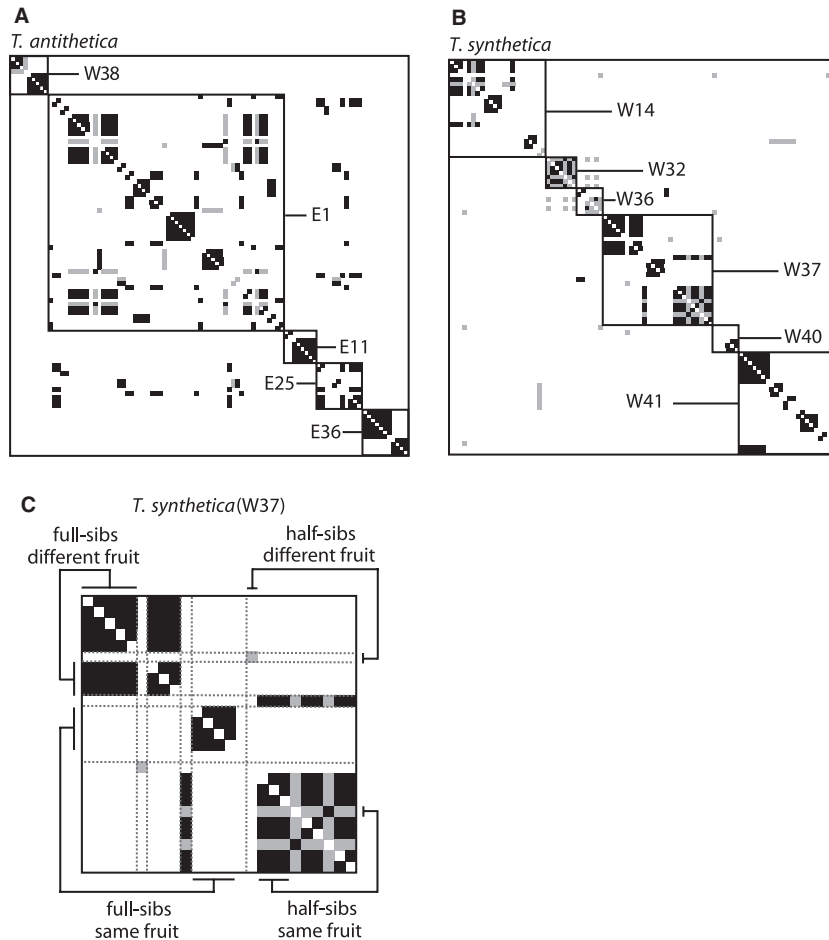


Fig. 4 Maximum likelihood estimates of pairwise sibship for representative samples of larvae collected within and among trees, for *T. antithetica* (A) and *T. synthetica* (B) larvae, based on combined data from microsatellite DNA genotypes and mtDNA haplotypes. Complete pair-wise sibship plots for the total sample of larvae are available as online supplementary material Fig S4. Each plot shows comparisons between pairs of individuals, with each cell (both above and below the diagonal) shaded according to the inferred relationship between them (black = full-sib, grey = half-sib, white = unrelated). The diagonals, comparing each larva to itself, are left unshaded. Individuals are ordered according to the tree and fruit from which each larva emerged, with larvae from the same tree enclosed by boxes (solid lines) along the diagonal. The tree from which each group of larvae was reared is indicated (E = eastern variety; W = western variety). (C) An example of larval sibships within and among fruit on a single tree; script annotations show example inferences of three different family structures.

consequence of being unable to reach the ovules, but would be less likely to damage the flower so badly as to prompt abscission.

The data presented here lend considerable support to this hypothesis and represent the first use of microsatellite DNA genotyping to estimate the fitness consequences of phenotype matching. Both moth species seem to pay a significant cost for oviposition on non-native hosts: *T. antithetica*, which has a short ovipositor and is smaller overall than its sister species, produces fewer larvae per oviposition event when laying eggs on the long-styled, western trees. This may suggest that the moth's shorter ovipositor is not able to reach all of the ovules. Likewise, the larger western moth, *T. synthetica*, seems to never successfully produce larvae on

short-styled eastern trees, suggesting that the moth's the larger ovipositor damages eastern flowers so badly as to induce abscission. Although individual moths might make-up for the lower larval survival on non-native hosts by spreading eggs across more flowers, moths dispersing between flowers and between trees likely experience elevated predation risk, and the data presented here show that female *T. antithetica* ovipositing on non-native western trees produced significantly fewer larvae overall (7.23 ± 6.98 larvae/female on eastern trees, vs. 2.5 ± 3.27 larvae/female on western trees, $P = 0.005$, two-tailed *t*-test), not just fewer larvae per fruit. Thus, matching between moth and floral phenotypes may mediate host specificity in this system. The findings therefore provide important empirical support

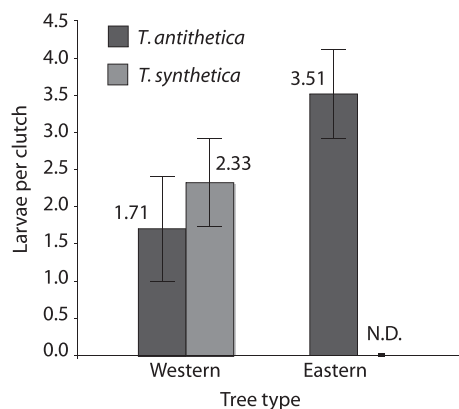


Fig. 5 Average clutch sizes (larvae reared per female per fruit) of *T. antithetica* and *T. syntheticca* moths on eastern and western tree varieties. Error bars show 95% confidence intervals about the mean.

for the prediction that natural selection acting on phenotype matching can, as a by-product, promote reproductive isolation in obligate pollination mutualisms.

It is important to note that the observed data might also be the product of other mechanisms that would maintain pollinator host specificity. For example, unpublished data show that flowers from the two tree varieties differ in their scent profile, which could allow the two moth species to preferentially orient towards preferred hosts (R. Raguso, personal communication). However, given that larval emergence rates on non-native hosts (*T. syntheticca* on eastern trees, and *T. antithetica* on western trees) were significantly lower than would be expected based on adult visitation rates, mechanisms other than olfaction must also influence host specificity. It is possible that even though adult moths apparently visit non-native hosts at high rates, they rarely attempt oviposition, or that, following oviposition, features of the floral tissue or secondary chemistry in each tree type favor the development of only native pollinator larvae. Last, as the data presented here considers only rates of larval emergence, and does not address the lifetime fitness consequences of oviposition errors, it is possible that other factors may affect the strength of selection acting on host specificity.

At this time, the features that act between a female moth's arrival at a flower and the production of larvae remain a black box, and the prediction that the two moth species will differ in performance on foreign hosts is not unique to the hypothesis that selection for phenotype matching promotes host specificity and reproductive isolation between tree types. Nonetheless, our data still provide an important test of this hypothesis; if we had found no differences in larval emergence rates it would suggest that mechanisms other than phenotype

matching determine host specificity and species boundaries in this system. Furthermore, although it is certainly the case that selection for matching between ovipositor and floral morphology is not the only process that could have produced the observed patterns, in the absence of concrete evidence that additional mechanisms determine oviposition success and larval survival, matching between the length of the female moth's ovipositor and the length of the floral style seems the most parsimonious explanation.

Ongoing studies in this system may soon allow us to better disentangle the mechanisms that mediate pollinator host specificity, and how host specificity may maintain reproductive isolation between tree types. It is at present difficult to distinguish consequences of phenotype matching from other confounding, host-related differences that may promote local adaptation. However, within the Tikaboo Valley contact zone trees that are intermediate in morphology occur at low, but measurable frequency. If these trees show continuous variation in style length, then by extending the methods used here to include intermediate trees, it may be possible to statistically partition the effects of phenotype matching from other host-related effects.

It is also unclear whether the differences in pollinator host specificity seen here are sufficient to explain the asymmetry in gene flow between host varieties identified previously. Whereas the patterns we have described in this study are qualitatively consistent with the chloroplast capture hypothesis, it is not clear whether the magnitude of nuclear gene flow from eastern trees into western trees is sufficient to explain the observed distribution of chloroplast haplotypes. Recently developed, biparentally inherited microsatellite markers for *Yucca brevifolia* may soon reveal whether pollinator mediated gene flow between tree types matches the differences in host specificity found here, and may make it possible to develop quantitative estimates of nuclear gene flow.

The Joshua tree system presents a rare and promising opportunity to uncover the role of reciprocal natural selection in promoting diversification in plant-pollinator mutualisms. The results of this study provide some of the first empirical support for the proposition that phenotype matching promotes speciation, and ongoing studies may soon allow improved quantification of natural selection acting on plant and pollinator phenotypes.

Acknowledgements

This project was funded by a grant from the National Science Foundation to OP and CIS (DEB 0516841). Audry Hite and Shantel Tank assisted with rearing of larvae from fruits and

DNA extraction for larval genotyping. The authors gratefully acknowledge this support.

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C.I.S. uses molecular tools to examine the role of ecological forces, such as coevolution and host–parasite interactions, in shaping population genetic processes and macroevolutionary patterns. C.S.D. is interested in the application of molecular genetics to questions involving the population structure and mating systems of plants and other organisms at shallow levels of evolutionary divergence. W.G. uses analytical and empirical approaches to find robust ways to study a species' niche. J.B.Y. uses molecular data and mathematical models to study the quantitative genetics of traits involved in coevolution. O.P.'s research interests focus on mutualism and coevolution.

Supporting information

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

Table S1 Summary statistics from nine microsatellite DNA loci genotyped for 501 larvae of *T. synthetica* ($n = 99$) and *T. antithetica* ($n = 402$), including number of alleles (N_A), expected heterozygosity (H_E), and observed heterozygosity (H_O)

Table S2 Matrilineal assignments for *T. antithetica* larvae as inferred using COLONY 2.0

Table S3 Matrilineal assignments for *T. synthetica* larvae as inferred using COLONY 2.0

Fig. S1 Simplified neighbor-joining tree showing relationships among mtDNA sequences from larvae collected in Tikaboo Valley, adults of known species status, and selected outgroups.

Fig. S2 Species identification of yucca moth larvae at the Tikaboo Valley contact zone, based on Bayesian clustering ($K = 2$) of microsatellite DNA data.

Fig. S3 Bayesian clustering for *Tegeticula* based on microsatellite DNA data.

Fig. S4 Maximum likelihood estimates of pairwise sibship for *T. antithetica* and *T. synthetica* larvae, based on combined data from microsatellite DNA genotypes and mtDNA haplotypes (black = full-sib, grey = half-sib).

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