Replicated host-race formation in bogus yucca moths: genetic and ecological divergence of *Prodoxus quinquepunctellus* on yucca hosts

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ABSTRACT

Goal: Assess host-race formation in certain moths by examining their genetic and ecological differentiation.

Organisms: Stalk-feeding moths, *Prodoxus quinquepunctellus*, collected from sympatric populations of *Yucca elata* and *Y. rostrata* in west Texas, USA.

Results: Moths on the two yuccas differed significantly in mtDNA haplotype frequencies, emergence time, wing dot number, body size, and ovipositor size and shape.

Conclusion: Host-race formation has probably occurred in this yucca moth although genetic divergence was low.

Keywords: diversification, host-race formation, mitochondrial DNA, ovipositor morphology, phenology, Prodoxidae, *Yucca*.

INTRODUCTION

Speciation as a consequence of ecological specialization has become increasingly accepted as a factor generating diversification among animals (Dieckmann and Doebeli, 1999; Kondrashov and Kondrashov, 1999; Schluter, 2001; Via, 2001; Kirkpatrick and Ravigné, 2002; Coyne and Orr, 2004), and empirical evidence as well as recent theoretical models suggest that such divergence of populations can occur in the absence of geographical isolation. Ecological specialization following host shifts has been suggested as an important factor behind the isolation of lineages in herbivorous insects, and many examples of host-race formation have been documented in recent decades (e.g. Bush, 1969; Carroll and Boyd, 1992; Emelianov *et al.*, 1995; Via, 1999; Groman and Pellmyr, 2000;

Nosil *et al.*, 2002; Thomas *et al.*, 2003; Diegisser *et al.*, 2004). Such host races can thus serve as models to test hypotheses about the factors driving local specialization that can lead to reproductive isolation and speciation. Empirical data show that both intrinsic and extrinsic factors are important in generating host-specific population differentiation, including phytochemistry (Becerra, 1997), phenology (Feder *et al.*, 1997; Groman and Pellmyr, 2000) and plant morphology (Carroll *et al.*, 1997), as well as parasitoid presence (Brown *et al.*, 1995; Feder, 1995; Thomas *et al.*, 2003) and predation on migrants (Nosil, 2004).

Although the number of documented cases of host-race evolution in herbivorous insects is growing quickly (Berlocher and Feder, 2002), few studies have addressed the frequency of replication of divergence within lineages, and whether such replication occurs through parallel or different trait divergence. Thus, the generality of host-race formation in closely related lineages with similar life habit remains poorly understood. One exception is the analysis of possible host-race formation in *Rhagoletis cerasi* (L.), a species belonging to the same genus as *R. pomonella* (Walsh), the major model of parapatric or sympatric speciation (Feder *et al.*, 2003; Schwarz *et al.*, 2003). Although *R. cerasi* flies feeding on two different hosts show phenological and behavioural differences, only one of six allozyme loci showed host-related differentiation, indicating little genetic divergence between populations utilizing different hosts (Schwarz *et al.*, 2003).

Here, we explore a second case of possible host-race formation in bogus yucca moths of the genus *Prodoxus* to test for parallel patterns of host-based divergence. The stalk-feeding *Prodoxus quinquepunctellus* (Chambers) was earlier recognized as a single species utilizing about 15 species of yuccas throughout the United States (Davis, 1967), but a recent phylogeographic survey by Althoff *et al.* (2001) demonstrated that the species in fact consists of two genetically and morphologically distinct lineages: the western *P. quinquepunctellus* and the eastern *P. decipiens* (Riley). In the course of that study, we noted that moths from *Yucca elata* (Engelmann) and *Y. rostrata* (Engelmann) showed distinctive phenotypes across their respective ranges. The phylogenetic tree based on mtDNA sequence data showed no evidence of divergence or unique haplotypes between moths feeding on the two hosts, but few specimens from these yuccas were included in that study (Althoff *et al.*, 2001). Based on observed divergence in flowering times of the hosts, and the corresponding phenotypic difference observed in the moths, we decided to pursue a finer-grained study in a site of sympatry of the two yuccas, so that climatic factors could be assumed to be equal.

In a previous study, Groman and Pellmyr (2000) showed that *P. decipiens* has colonized the introduced *Yucca aloifolia* (L.) in historical time where it co-exists with the native *Y. filamentosa* (L.) along the southeastern US Atlantic coast. The derived host race shows divergence in adult emergence phenology and body size; the latter is scaled to the size of the elaborate saw-like ovipositor used by the female to deposit eggs into host inflorescence stalks. Genetic analyses did not show divergence at the mitochondrial nucleotide level, but divergence was detected in allozyme frequencies. Here we test whether similar divergence has occurred in *P. quinquepunctellus* (Althoff *et al.*, 2001), a species that utilizes two parapatric yucca species in part of its range. In contrast to the original study, where host sympatry has only occurred for at most a few hundred years, the two hosts of *P. quinquepunctellus* have likely co-existed in the study area for at least 12,000 years (Wells, 1966), and potentially been parapatric on a time scale of hundreds of thousands of years. Specifically, we tested whether similar morphological host-race formation has occurred in this older ecological setting.

MATERIAL AND METHODS

Biology of the moths

Prodoxus quinquepunctellus (Fig. 1A) is widely distributed in the western United States and adjacent Mexico, and has been documented to feed on seven capsular-fruited species of yuccas, including *Y. elata* and *Y. rostrata* (Althoff *et al.*, 2001). The nocturnally active adults reside in the flowers during the day, and mating takes place within yucca flowers. The female uses a puncturing-sawing behaviour to insert eggs under the epidermis of inflorescence stalks during the bud or early flowering stage. As the stalk hardens with flowering progression, the female is occasionally able to puncture the surface but she cannot retract the ovipositor (Riley, 1882), thus there is a very short time window for successful oviposition. The larva feeds within the stalk for about a month before entering diapause. Following one or more years of diapause, pupation occurs inside the stalk, and the emergence of the
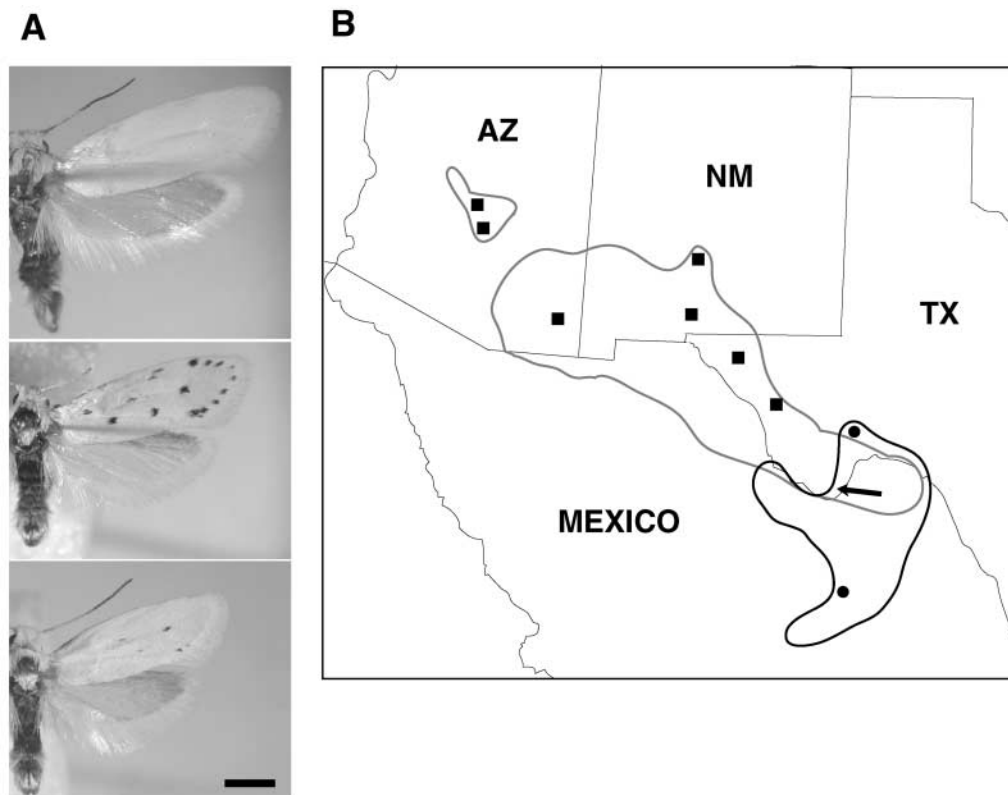
Fig. 1. (A) Wing pattern of *Prodoxus quinquepunctellus* from *Y. elata* (top) and *Y. rostrata* (middle and bottom). Scale bar is 2 mm. (B) Distribution of *Yucca rostrata* (black line) and *Y. elata* (grey line). Dots show known sites of moth infestation on *Y. rostrata* (NE Ft Stockton, TX; NE San Pedro de las Colonias, Coahuila, Mexico). Squares show known sites of moth infestation on *Y. elata* (AZ: Cottonwood, Camp Verde and Willcox; NM: Jornada and Magdalena; TX: Sierra Blanca and Valentine). The arrow indicates the study site with moth infestation on both hosts. AZ: Arizona; NM: New Mexico; TX: Texas.

short-lived adult moths coincides with the flowering period of their host (Davis, 1967; Groman and Pellmyr, 2000). The impact of stalk-feeding by *Prodoxus* on host fitness has been found to be non-significant, so the interaction may be considered a commensalism (Althoff *et al.*, 2004).

Biology of the two yucca species

Yucca elata and *Y. rostrata* are capsular-fruited yucca species, but phylogenetically widely separated. *Yucca rostrata* is part of the series *Rupicolae*, a basal cluster of species that inhabit well-drained limestone slopes, whereas *Y. elata* is part of the derived Chaenocarpa that shows less edaphic specificity and generally inhabits grass flats or desert plains (Clary, 1997); it inhabits the desert floor in the study area. *Yucca elata* is widespread within the Mexican Highlands geomorphic province of the northern Chihuahuan desert, in essence in the Rio Grande/Rio Bravo basin (Fig. 1B). *Yucca rostrata* occurs in high desert areas of northern Mexico, extending into the Big Bend region of western Texas (Fig. 1B). Both species produce one or more inflorescences on separate stalks, each consisting of up to a few hundred white flowers. The stalk of *Y. elata* is on average thicker and taller than that of *Y. rostrata*. Flowering time dovetails between the two species, with *Y. rostrata* effectively completing flowering as it commences in *Y. elata*. In the Big Bend region, *Y. rostrata* usually flowers from mid-April to early May, whereas *Y. elata* flowers from early May to early June. Because of the habitat differences, the species are mostly spatially segregated, but grow in sympatry on lower slopes that connect the desert floor with local mountain ranges. Even without this transition zone, the distance between host species is certainly within flight range of the moths.

Collection of moths

Adult females and larvae of *P. quinquepunctellus* from *Y. elata* and *Y. rostrata* used for phenological, morphological and molecular analyses, were collected in Big Bend National Park, Texas (29°33′N, 102°57′W) during 1994, 2000, 2001 and 2004 (Fig. 1B). This is the only documented site where *P. quinquepunctellus* feeds on sympatric populations of the two hosts, and therefore the only location where the hypothesis of host-associated divergence in this moth can be tested accurately.

Moth emergence

A total of eight old *Y. elata* and 22 *Y. rostrata* inflorescence stalks with confirmed *Prodoxus* presence were collected in 2004 in Big Bend National Park at Tornillo Flat and Dagger Flat, respectively. The collection sites are separated by 175 m in altitude and are 12 km apart, but the intervening area holds contiguous stands of the two species, which can be seen growing side by side. Although larvae have been found in stalks in the zone of overlap between the yucca species during previous years, very few larvae were found in stalks in that area during 2004. Upon return to the laboratory, stalks were placed in 1 mm mesh cages under identical climate conditions (16:8 h light:dark, 35% relative humidity, 25:20°C day:night temperature) to induce eclosion of moths. Adults were removed from cages on a daily basis to record emergence dates between moths from the different hosts, and difference in emergence times between host types was analysed using an unpaired *t*-test.

Morphology

Female moths used for morphological measurements were collected on their hosts (1994, 2000 and 2001) or following emergence from host stalks in the laboratory (2004), thereby assuring that variation among host plants and locations was well represented. In total, 20 moths from *Y. elata* and 25 moths from *Y. rostrata* were used. The wings and abdomen were removed from each female and kept as voucher. The length of the forewing was used as a character to estimate body size. In addition, the number of black dots on the forewing was counted. To measure ovipositor traits, the abdomen was boiled in 10% KOH for 3 min to remove adipose tissue and to facilitate dissection. The same four ovipositor traits used in Groman and Pellmyr (2000) were measured in this study: length of posterior apophyses, height and length of ovipositor tip, and number of ovipositor notches (Fig. 2A, B). Examination of morphological characters was performed using an Olympus SZ-PT dissecting microscope equipped with an ocular micrometer. A principal component analysis (PCA) was performed based on all traits, except the number of forewing dots, to test for an overall difference in morphology between moths from the two hosts. Differences in forewing dot number were analysed using Wilcoxon's signed ranks test, and the remaining traits were analysed using unpaired *t*-tests. Before statistical analysis, the data were checked and
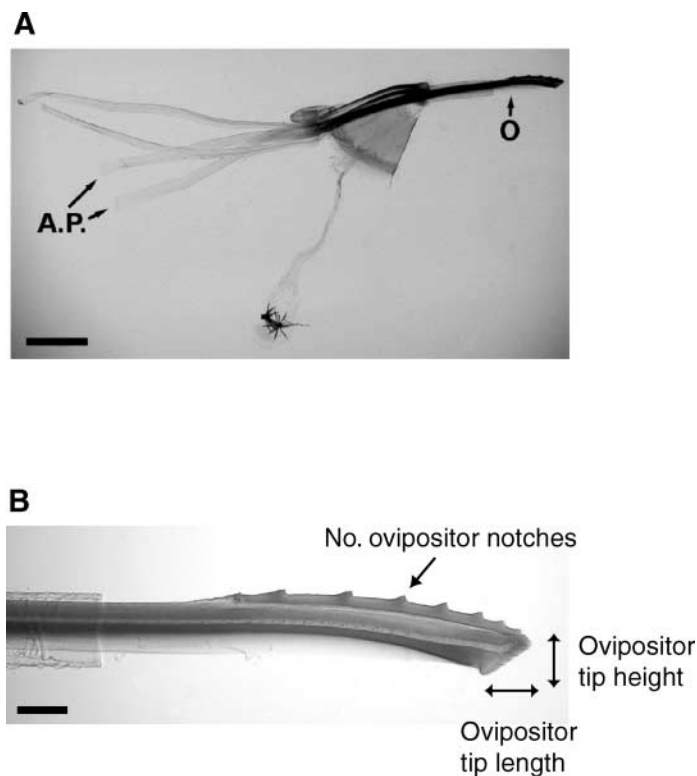
Fig. 2. Female ovipositor characters measured on *P. quinquepunctellus* in this study. (A) A.P. = apophyses posteriores, which terminate as the fused ovipositor sheath (O). Scale bar is 1 mm. (B) Tip of ovipositor. Scale bar is 0.1 mm.

corrected as needed for normality and equal variances. Statistical tests were performed using JMP 3.2.1 (SAS Institute, 1997).

DNA sequencing

Mitochondrial DNA sequence variation in moths was examined using 48 specimens from *Y. elata* and 47 specimens from *Y. rostrata*, collected in 1994, 2000 and 2001. Total DNA was extracted from the thorax and abdomen of adult moths and from whole larvae using the IsoQuick DNA isolation Kit (Orca Research, Inc., Bothell, WA). The polymerase chain reaction (PCR) was then used to amplify a 789-bp fragment of the cytochrome oxidase subunit I gene and transfer RNA leucine gene. The protocol was the same as used by Althoff *et al.* (2001). Each reaction volume of 30 μl included 10× PCR buffer, 1.67 mM MgCl$_2$, 0.2 mM dNTPs, 0.033 units of Taq polymerase, 0.33 mM of each primer, and approximately 100 ng of moth DNA. Primer sequences were 2231F: (5′-CCAGGATTTGGTATAAATTTC-3′) and 3020R: (5′-GTAATGGATTTAAGCCCCT-3′), with numbers referring to nucleotide positions in the *Drosophila yakuba* mitochondrial genome (Clary and Wolstenholme, 1985). The PCR conditions were: 1 cycle at 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, 50°C for 45 s, and 72°C for 1.5 min. Products were cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequencing products were then generated using approximately 50 ng of Qiagen-column purified PCR product, BigDye terminator cycle sequencing mix (PE Applied Biosystems), and 4 pmol of primer. The PCR conditions were 25 cycles at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. Centri-sep Sephadex columns (Princeton Separation, Adelphia, NJ) were used to clean products, which were later sequenced in both directions using an ABI 3730 automated capillary sequencer (Applied Biosystems, Inc., Foster City, CA). Forward and reverse sequences were checked with Sequencher 3.1 (Gene Codes Corp., Ann Arbor, MI) and aligned in PAUP* ver. 4.0b4a (Swofford, 2000). We used analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) to test for genetic differentiation between the two host types. This analysis incorporates both sequence divergence and haplotype frequencies to partition the genetic variance among hierarchical groups.

RESULTS

Moth phenology

A total of 35 moths from *Y. rostrata* and 20 moths from *Y. elata* emerged from stalks (Fig. 3). Moths from *Y. rostrata* emerged significantly earlier than those feeding on *Y. elata* ($t_{53} = 11.95$, $P < 0.001$). The mean emergence time differed by 2 weeks between host types and there was only one day of overlap between the moths from the two hosts. This pattern of moth emergence corresponded to the flowering phenology of their hosts.

Morphology

The two principal components with eigenvalues >1 explained 86% of the variation in morphology (Fig. 4). The first principal component showed similar positive loading for all traits except the number of ovipositor notches, which had negative loading, and size explained 65% of the overall variation in morphology. Much of the difference observed in
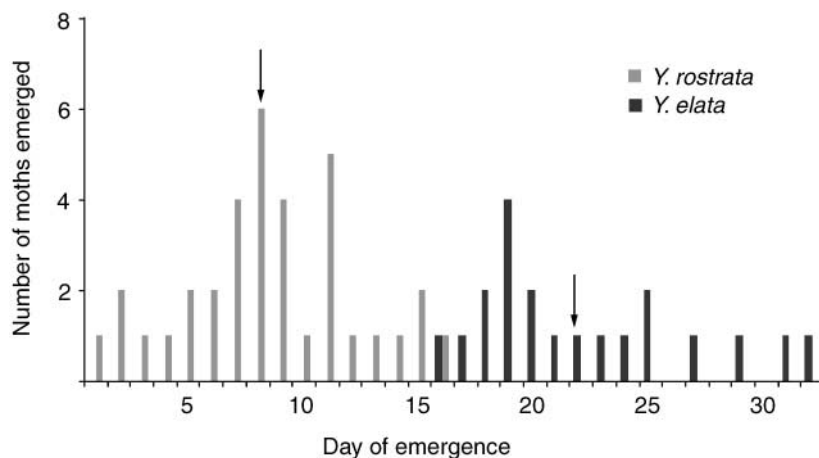
Fig. 3. Emergence of *P. quinquepunctellus* from stalks of different yucca hosts under controlled climatic conditions. Arrows indicate the mean emergence date for each host type.
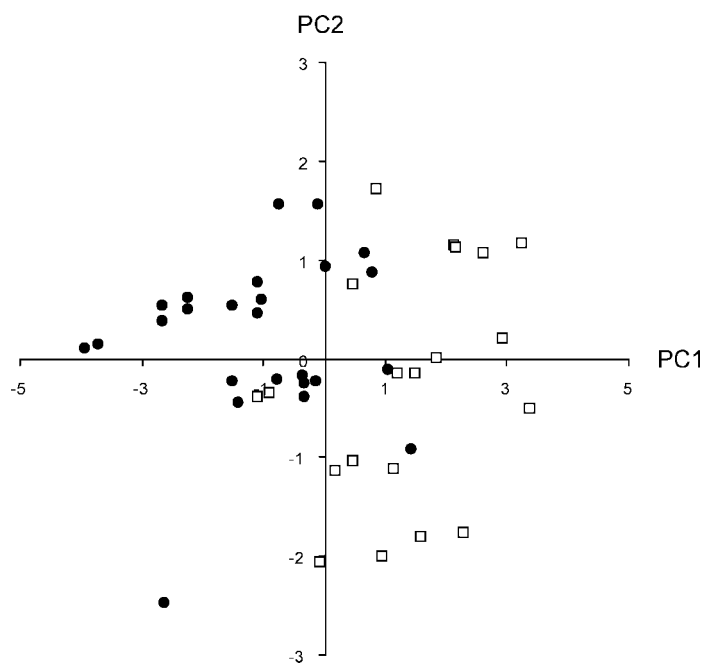
Fig. 4. Score plot of the first two principal components, PC1 (x-axis) and PC2 (y-axis), based on five morphological characters of female *P. quinquepunctellus* from *Y. rostrata* (●) and *Y. elata* (□).

ovipositor morphology was indeed driven by divergence in body size of moths from the two yucca species. Females originating from *Y. rostrata* had smaller wings and more forewing dots than females from *Y. elata* (Fig. 5). The length of the posterior apophyses, the height of the ovipositor tip and the length of the ovipositor tip were also less for females on *Y. rostrata* than those on *Y. elata*. In contrast, females on *Y. rostrata* had more notches
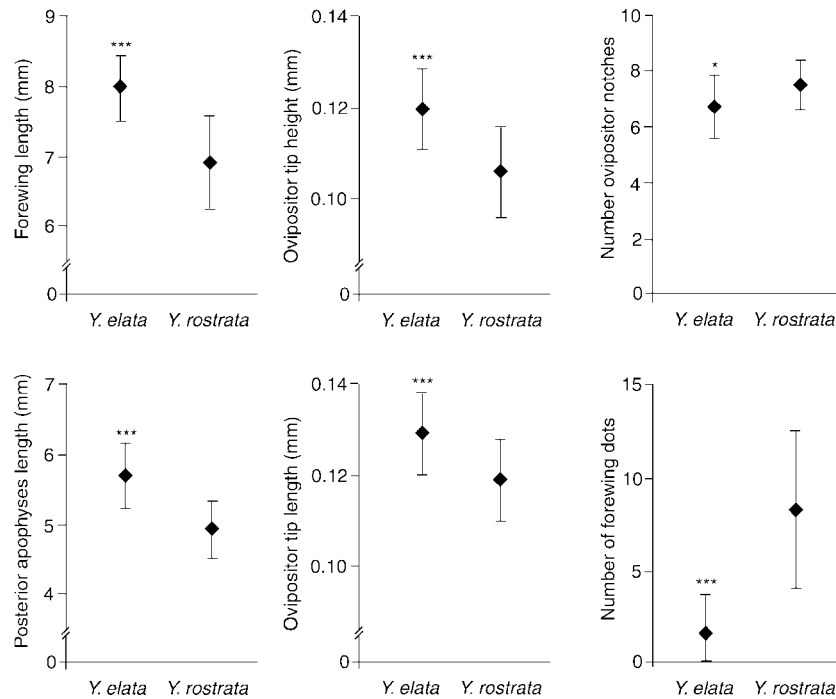
Fig. 5. Morphological characters (mean ± standard deviation) of female *P. quinquepunctellus* collected from *Y. elata* ($n = 20$) and *Y. rostrata* ($n = 25$). * $P < 0.05$, *** $P < 0.001$.

on their ovipositors than females on *Y. elata*, in spite of their smaller size (Fig. 5). All morphological characters had overlapping distributions between moth populations, and there was no single diagnostic trait that distinguished the two host types.

mtDNA haplotype frequencies

From the amplified region, 618 base pairs were readable in both directions in all samples. Seven unique haplotypes were found among the 95 individuals analysed. The two most abundant haplotypes were found in 44% and 19%, respectively, of the individuals analysed, whereas the remaining haplotypes occurred in frequencies <10% (Table 1A). Three haplotypes were present in both host types, two were only found in *Y. elata* moths, and the remaining two were specific to *Y. rostrata* moths (Table 1A). Sequence divergence was low (0.49% uncorrected divergence) with a maximum of three substitutions. Analysis of molecular variance revealed a significant effect of yucca host type on the partitioning of genetic variance (Table 1B), with 18% being attributable to the host.

DISCUSSION

Phenological divergence

As the coordination of an insect's life cycle with the availability of essential resources is critical for survival and reproduction, phenological differences between host plants could be

Table 1. (A) Distribution of mtDNA cytochrome oxidase I sequence haplotypes found between host types of *P. quinquepunctellus* in this study. (B) AMOVA table demonstrating partitioning of genetic variance in mtDNA cytochrome oxidase I sequence data between host types

(A)

Haplotype	No. in *Y. elata* moths	No. in *Y. rostrata* moths	Total
A	11	31	44
B	18	0	18
C	8	1	9
D	3	6	9
E	8	0	8
F	0	8	8
G	0	1	1
Total	48	47	95

(B)

Source of variation	d.f.	Sum of squares	Variance components
By yucca species	1	6.24	0.12 Va
Within populations	93	48.87	0.53 Vb
Total	94	55.11	0.65

$\varphi_{ST} = 0.19$***

*** $P < 0.0001$.

an important factor promoting divergence among populations of herbivorous insects that feed on different hosts (Feder *et al.*, 1997; Berlocher and Feder, 2002; Thomas *et al.*, 2003). In the present study, *P. quinquepunctellus* originating from two yucca hosts showed distinct emergence times in the laboratory, with only a single day of overlap, and the time of eclosion corresponded to the relative flowering time of their hosts (Fig. 3). Moths feeding on *Y. rostrata* growing on mountain slopes emerged earlier than those feeding on *Y. elata* growing on the desert floor, in spite of the presumably lower temperatures at higher altitudes. Such a pattern can most likely be explained as an adaptation by moths to match the phenology of their host, rather than as an effect caused by a difference in temperature between the collection sites. By comparison, in *P. decipiens*, there was a gap of 6 days in emergence between the two host races when reared under climate-controlled conditions (Groman and Pellmyr, 2000). Thus, the difference in emergence time, coupled with mating on the host plant, can translate into allochronic isolation between host-feeding types.

The observed difference in emergence of moths from the two hosts may in fact be even more pronounced in the field, with local differences in climate, compared with the controlled rearing environment provided in this study. Such effects have been observed in other studies comparing field and laboratory data on insect emergence in yucca moths and other insects (e.g. Groman and Pellmyr, 2000; Thomas *et al.*, 2003). Because *P. quinquepunctellus* originating from the two hosts were exposed to identical climatic conditions (light, temperature and humidity) in our rearing experiment, the pattern observed suggests a genetic component for differences in timing of emergence between host types, as appears to be the case also in *P. decipiens*. This path of diversification may be particularly pronounced in herbivores such

as *Prodoxus*, which utilize ephemeral plant parts only suitable for oviposition during a brief period.

Morphological divergence

Differences in fitness-related morphological traits among populations of phytophagous insects may be a result of divergent selection on different hosts (Carroll and Boyd, 1992; Carroll *et al.*, 1997). For example, high diversity in ovipositor characters observed within *Prodoxus* (Davis, 1967; Pellmyr *et al.*, in press) has been suggested to reflect adaptive diversification among moth species to facilitate insertion of eggs into various parts of their yucca hosts (Groman and Pellmyr, 2000). In this study of *P. quinquepunctellus*, female ovipositors were significantly thicker in moths feeding on *Y. elata* than in moths feeding on *Y. rostrata*, and *Y. elata* moths were also larger than those on *Y. rostrata* (Fig. 5). However, in spite of their smaller size, females from *Y. rostrata* had more notches on their ovipositors than those from *Y. elata*. In fact, when controlling for difference in body size, *Y. rostrata* moths had on average 30% more notches on their ovipositors than those from *Y. elata*. This observation is unlikely to be explained by allometric effects, but may instead reflect adaptations in females to facilitate insertion of eggs into stalks of their respective host.

In *P. decipiens*, a similar pattern is present, with moths on *Y. aloifolia* having thinner ovipositors than moths on *Y. filamentosa*, when controlling for difference in body size. Groman and Pellmyr (2000) speculated that a thin ovipositor may be advantageous for insertion of eggs into the stalk of the fleshy-fruited *Y. aloifolia*, which is softer than that of the capsular-fruited *Y. filamentosa*. Stalk hardness of the two capsular-fruited yuccas in the present study has not been formally measured, but the stalk of *Y. elata* is generally thicker and harder to rupture than that of *Y. rostrata* (G.P.S. and O.P., personal observations).

The morphological divergence observed among the two host types in *P. quinquepunctellus* is mainly attributable to variation in body size, except the number of notches on the ovipositor. This result suggests that host-associated differentiation among *P. quinquepunctellus* populations may be largely due to allometric effects associated with changes in body size. For *P. decipiens*, Groman and Pellmyr (2000) also demonstrated significant differences in body sizes between the two host races. Quantitative genetic studies are needed to determine if selection is acting on body size directly, or if body size changes due to its tight coupling to another morphological trait under selection. A plausible speculation would be that selection on body size is an indirect effect of selection on ovipositor size, which in turn determines oviposition success in different substrates. The apophyses, which are the structures that control extrusion and retraction of the ovipositor, are fully enclosed within the abdomen, and generally abut the anterior end, so any selection for longer apophyses requires a larger abdomen.

Genetic divergence

The analysis of molecular variance revealed a significant difference in mtDNA haplotype variation between *P. quinquepunctellus* populations collected from sympatric *Y. elata* and *Y. rostrata* (Table 1). Whereas some host-specific haplotypes were documented, most variation was due to differences in haplotype frequency rather than sequence divergence. In host races of the sister species *P. decipiens*, no divergence was detected among populations feeding on the ancestral *Y. filamentosa* and the derived *Y. aloifolia*, when using the same sequence

region and statistical approach (Groman and Pellmyr, 2000), but the number of haplotypes was far lower on the novel host, consisting mostly of the most common haplotype in the ancestral host, suggesting a recent genetic bottleneck or rapid isolation. Meanwhile, data from more rapidly evolving allozymes detected significant differences between the two host strains and within the younger strain. As the host shift in *P. decipiens* is very recent (< 500 years), a significant divergence between host-feeding populations would not be expected for the COI gene, which has a moderate rate of sequence evolution for yucca moths (Pellmyr and Leebens-Mack, 1999; Althoff *et al.*, 2001). Because the yuccas used in this study have co-existed for a very long time, it would follow that if the morphological, biological and genetic differences detected reflect consistent isolation on the two hosts, genetic divergence should have proceeded much farther in *P. quinquepunctellus* than in *P. decipiens*. This was not found, and the relatively low level of sequence variation observed in this study (0.49%) also was comparable with a maximum divergence of 0.58% for the host races of *P. decipiens* (Groman and Pellmyr, 2000). However, moths were only analysed from a single site in the present study, and data from additional *P. quinquepunctellus* populations feeding on the two yuccas are needed to confirm whether the pattern of low sequence variation holds true also on a larger geographic scale.

Replicated host-race formation in stalk-feeding yucca moths

We have documented a second case of host-race formation within the genus *Prodoxus*, and found that the same traits – adult emergence time, body size and female ovipositor morphology – differentiate between ecologically divergent lineages in *P. quinquepunctellus* and *P. decipiens* (Groman and Pellmyr, 2000). The concordance between the two lineages suggests that host-race formation may be relatively simple in a proper ecological context; covariance of body size and functionally critical ovipositor traits suggest that selection on morphology and emergence time may suffice to generate diverging lineages in this case. In the case of *P. decipiens*, where the younger host can be identified, selection has been for smaller body size. In *P. quinquepunctellus*, the older host cannot be identified, because the two yucca hosts are confined to the Chihuahuan desert. The desert has probably existed for at least 6 million years (Riddle, 1995), and the phylogenetic information available for the capsular-fruited yuccas also suggests very old age for both species. In fact, packrat middens from caves currently surrounded by *Y. rostrata* in the Big Bend region contain fragments of *Y. rostrata* that are about 12,000 years old (Wells, 1966). *Yucca elata* is unlikely to be found in middens for taphonomic reasons; it grows on adjacent desert floor, where middens are exposed to weather and relatively quickly destroyed. Meanwhile, the vegetation profile reconstructed from the paleodata of the area resembles extant communities that include *Y. elata* (Wells, 1966).

Whereas the exact duration of co-existence of yuccas is uncertain, it is obvious that it extends much farther back in time than that of *Y. filamentosa* and *Y. aloifolia*. Nonetheless, the magnitude of genetic divergence between the host races in the east and in the west is not very different. This would suggest that the host races found in the Big Bend region diverged quite recently, and are not nearly as old as the hosts. One possible explanation might be that these host races tend to be transient on longer time-scales, being lost to extinction or admixture in response to factors such as climate fluctuations. This would hold true even if the co-existence of the host races in Big Bend reflects secondary contact of lineages evolved elsewhere. If correct, we may be able to document many separate origins of host races in different parts of the sympatric range, where traits should converge across origins but

different neutral genetic markers will characterize the separate origins. Understanding the persistence and fixation of evolving host-specific lineages will be critically important in weighing the role of ecological versus allopatric factors in speciation among insect genera with different life-history traits.

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