



Divergent host plant adaptation drives the evolution of sexual isolation in the grasshopper *Hesperotettix viridis* (Orthoptera: Acrididae) in the absence of reinforcement

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Early stages of lineage divergence in insect herbivores are often related to shifts in host plant use and divergence in mating capabilities, which may lead to sexual isolation of populations of herbivorous insects. We examined host preferences, degree of differentiation in mate choice, and divergence in cuticular morphology using near-infrared spectroscopy in the grasshopper *Hesperotettix viridis* aiming to understand lineage divergence. In Kansas (USA), *H. viridis* is an oligophagous species feeding on *Gutierrezia* and *Solidago* host species. To identify incipient mechanisms of lineage divergence and isolation, we compared host choice, mate choice, and phenotypic divergence among natural grasshopper populations in zones of contact with populations encountering only one of the host species. A significant host-based preference from the two host groups was detected in host-paired feeding preference studies. No-choice mate selection experiments revealed a preference for individuals collected from the same host species independent of geographic location, and little mating was observed between individuals collected from different host species. Female mate choice tests between males from the two host species resulted in 100% fidelity with respect to host use. Significant differentiation in colour and cuticular composition of individuals from different host plants was observed, which correlated positively with host choice and mate choice. No evidence for reinforcement in the zone of contact was detected, suggesting that divergent selection for host plant use promotes sexual isolation in this species. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, 100, 866–878.

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INTRODUCTION

Strong associations develop between an insect herbivore and its host plant both as food and primary habitat, accentuating the likelihood that local adaptation will occur and initiate a chain of events leading to phenotypic and genetic differentiation (Mopper & Strauss, 1998; Berlocher & Feder, 2002; Gavrillets & Vose, 2009). Host shifts cause behavioural differences

in feeding and segregation of insects feeding on different host species, which then promote speciation in animals by reducing mating opportunities between individuals of different host plants (Egan, Nosil & Funk, 2008; Nosil, Egan & Funk, 2008). Feeding specialization often sets the stage for reduction in mating between populations feeding on alternate hosts (Via, 1999), and reinforcement of divergence can occur when hosts have the potential to come into contact rather than when in allopatry. These differences may or may not reflect reinforcement of mating isolation by character displacement. For mobile,

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differentiating populations capable of interbreeding, reinforcement may be important in maintaining assortative mating (Funk, 1998). Mechanisms acting to reinforce mating isolation include selection against hybrids, lower fertility, and higher mortality in inter-specific mating (Butlin, 1989, 1995; Noor, 1999; Servedio & Noor, 2003; Servedio, 2005).

Diet specialization by insect herbivores occurs in response to ecological, physiological, behavioural, and evolutionary forces (Fox & Morrow, 1981; Berenbaum & Zangerl, 1998). Specialization can occur as a result of constraints from physiological and biochemical trade-offs that limit performance on alternate hosts (Joshi & Thompson, 1995; Berenbaum & Zangerl, 1998). Even in sympatry, insect herbivore populations often exhibit fine-scale genetic differentiation in association with particular host plant species (Mopper, 1996). Increased performance on local host plants could reinforce local adaptation and lead to host races (Joshi & Thompson, 1995; Thomas & Singer, 1998). This intense association with the host plant thus makes insect herbivores susceptible to disruptive selection if and when they change hosts (Mopper & Strauss, 1998; Berlocher & Feder, 2002; Feder *et al.*, 2003; Nosil, 2005; Nosil, 2007).

The proliferation of host races may result from geographic isolation or from interactions among local factors such as relative host availability or competition. These selective pressures lead to local adaptation associated with the evolution of host shifts and strong selection for preferences. For some species, host race formation and speciation is a result of tension between plasticity in the diet, which allows for shifts to occur, and specialization in the diet, which allows for host race integrity. Some herbivores may have the ability to feed on an array of host plant species. This plasticity in host choice may lead to niche separation that segregates mate choice interactions and promotes divergence in herbivores using different host plants (Gorur, Lomonaco & Mackenzie, 2005). Such specialization in the face of phenotypic plasticity would be enhanced in highly philopatric species prone to living on one or several host plants almost exclusively.

The switch to an alternate host may affect organismal traits that can feed back and affect divergence. For example, diet composition can influence the abundance and composition of hydrocarbons in the cuticle (Chapman, Espelie & Peck, 2000). Differences in cuticular attributes likely play a role in communication and mate selection during courtship, thus contributing to the process of host race formation if divergence of cuticular signals leads to nonrandom mating between populations feeding on distinct host plants (Neems & Butlin, 1994). Indeed, cuticular hydrocarbons are used in taxonomic studies to differentiate among species, including Isoptera (Haverty

et al., 1988), Diptera (Phillips *et al.*, 1990), Coleoptera (Lockey & Metcalfe, 1988), and Orthoptera (Chapman *et al.*, 1995, 2000). Variation in cuticular composition accompanied by assortative mating has been hypothesized to lead to pre-mating reproductive isolation (Tregenza, Pritchard & Butlin, 2000).

Character displacement can also reinforce pre-mating isolation, where assortative mating and morphological differentiation are stronger in areas of sympatry than in allopatry. Because selection for reinforcement occurs in sympatry where both host plants are present, greater selection for mating preference should be detected in areas of secondary contact compared to allopatric areas. This has been demonstrated in mate choice studies and hybrid viability assays in walking sticks, *Timema cristinae*, and fruit fly, *Drosophila pseudoobscura* (Noor, 1995, 1999; Nosil, Crespi & Sandoval, 2002). Alternately, if philopatric populations remain on one host plant with little dispersal, reinforcement by character displacement may not be necessary to keep populations mating assortatively.

Hesperotettix viridis (Thomas) is an oligophagous grasshopper species that feeds on composites (Asteraceae) throughout its broad geographic distribution (Joern, 1979, 1985; Traxler & Joern, 1999). Individuals spend most of their time feeding, thermoregulating, avoiding predators, and mating on the same host plant (Parker, 1983; Pfadt, 1994; Traxler and Joern, 1999). In Kansas, we observed two distinct *H. viridis* phenotypes, where eastern populations encounter only *Solidago* and western populations encounter only *Gutierrezia*. Some *H. viridis* populations occur at geographically intermediate locations where both host plant populations co-occur.

Recently, Grace, (2009) reported genetically-based incipient host race diversification in *H. viridis* populations in Kansas consistent with that expected for host races. These populations fit the definition of a host race complex: each host race population exhibited strong host fidelity, coexisted in sympatry in at least part of its range, was genetically differentiated at more than one locus, and was spatially and temporally replicable (Dre's & Mallet, 2002). Reciprocal transplant experiments under field conditions in Nebraska revealed divergence in ecological performance and host preference by *H. viridis* in sympatric populations, including decreased fitness in mismatched host plant pairings (Traxler & Joern, 1999). Such physiological responses to host plant use could isolate host-based populations and reinforce the evolution of diet specialization.

In the present study, we assessed the relative contribution of mate selection (sexual isolation), host choice (feeding choice) and reinforcement of those phenomena to the creation of host races of *H. viridis*. In support of host race formation by *H.*

viridis, we hypothesized that: (1) grasshoppers would prefer the 'home' host plants to 'alternate' host plants if host plant use drives ecological divergence; (2) individuals from the same host plants would prefer to mate with one another rather than with those from different host plants; and (3) differences in host plant use and sexual isolation translates into morphological and physiological differences in animals from different host species. Because of the sedentary nature of *H. viridis* as well as its propensity to feed on multiple host taxa across a broad geographic distribution, reinforcement of these behavioural and physiological differences is not necessarily expected. Although character displacement for greater feeding specificity would be expected among the host plant groups in regions where they co-occur in comparison to allopatric distributions, these races may segregate on a very fine spatial scale, where reinforcement by character displacement does not occur. In such a microallopatric scenario, host plant specificity could lead to host races not encountering each other even in syntopic distributions. If reinforcement should occur, we expect greater choosiness in mate selection and feeding preferences in the regions of syntopy than in allopatric regions.

MATERIAL AND METHODS

SAMPLE COLLECTION

Hesperotettix viridis is patchily distributed in Kansas. Populations of *H. viridis* were collected from areas where potential hosts from the plant genera *Gutierrezia* and *Solidago* coexisted (zone of syntopy) and from areas with pure stands of only one of the two host plant taxa (*Gutierrezia* to the west and *Solidago* to the east of the zone of syntopy). Syntopy refers to species or host plants co-occurring at the same site whereas in sympatry two species co-exist in the same region (Rivas, 1964). At each of eight populations (four from *Gutierrezia* and four from *Solidago*, referred to as East, West, and Central regions) 40–60 individuals were collected from each site in 2008 (see Supporting information, Table S1). *Hesperotettix viridis* individuals were collected from their respective host plants by hand after recording the activity of the respective insect on its host plant (feeding or resting on host plant). Live individuals were collected as later-stage nymphs or recently-emerged teneral adults, returned to the laboratory, and reared until adult emergence. Sexes were kept isolated in separate cages and were fed material from the plants species from which they were collected.

FEEDING TRIALS

Choice assays were conducted. A total of 299 insects from eight populations were provided simultaneously

a choice of both *Gutierrezia* and *Solidago* plants. Individual insects were placed in a ventilated clear plastic arena (18 × 13 × 10 cm) with one approximately 10-cm cutting from each of the two host plants, the bottom of which was placed in small plastic vials filled with water to keep the plants fresh for the duration of the feeding trials. Feeding assays were initiated in the morning and conducted for 8 h. Insects were then left overnight in the container and fasted for 24 h prior to commencing remaining feeding trials. Feeding activity was recorded every 30 min for 8 h. Individuals were used only once in the feeding trials. Most insects fed only on *Gutierrezia* or *Solidago*, although some individuals (< 4%) fed on one host plant during one observation interval and the alternate host in the second observation interval. The observations were summarized as 0 or 1 for each insect that fed on *Gutierrezia* and *Solidago*, respectively. Observations for the few insects observed feeding on multiple hosts were characterized based on the host species on which they spent the majority of the time.

MEASUREMENT OF CUTICULAR CHARACTERISTICS

Near-infrared spectra (NIR) were used to quantify and compare cuticular absorbance at specific wavelengths between individuals from different hosts. This technique measures variability in structural components of the cuticle, and variation in visual characteristics in the two host forms. Near-infrared spectra of individuals from each of the eight populations were collected. Spectra (450–2500 nm) were collected from the thorax of each grasshopper using an ASD QualitySpecPro Benchtop Spectrophotometer (Analytical Spectral Devices). The NIR spectra were recorded at 2-nm intervals as absorbance (log 1/Reflectance) mode in the 450–2500 nm range using the RS3 software (Emerson Process Management). Individuals were positioned dorsal side down and the samples were illuminated with light from a bifurcated fibre optic reflectance probe (diameter 4.8 mm) positioned 5 mm from the bottom of the viewing area. Because males in *H. viridis* are smaller than females, males and females were analyzed separately to account for any sex-based variability, and the results were weighted to account for unequal sample numbers from each host. The instrument was optimized and a new baseline was determined at the beginning of each set of population samples.

MATING TRIALS

No-choice mating trials were conducted by placing a combination of one male and one female from the same host or alternate host into a clear plastic arena

as was used in the feeding study. Observed mating activity was recorded every 30 min for 8 h, after which the insects were left in the plastic arena overnight in preparation for paired-choice tests. Each individual was only used once in the mating trials. Individuals were used 5–10 days after the imaginal moult in mating studies. Observations were summarized as 0 for not-mated and 1 for mated pairs. The experiment was separated into two groups of four populations each, and all pairwise mating combinations were conducted. Ten pairwise combinations of mating trials were conducted for each group of which four included combinations of individuals from different hosts, and six trials were same-host combinations within a group. Each pairwise combination of mating trials was replicated 16 times; males collected from *Gutierrezia* were paired with females from *Solidago* for the first eight trials, and females from *Gutierrezia* paired with males of *Solidago* for the next eight trials. A total of 320 mating trials (16 replicates \times 20 crosses) were conducted.

Paired-choice mating trials were conducted the day after completion of no-choice experiments for: (1) trials where insects mated with the individuals from the other host plant (cross matings) and (2) trials where insects did not mate when the possible mate was from the alternate host. An equal number of mating pairs were used ($N = 18$ each for trials 'a' and 'b'). A male from the same host plant as the female was added (in addition to the male already present from the different host) to the clear plastic arena and observations were recorded as for the no-choice trials. Trial 'a' tested mating propensity when a choice was available to determine if an insect from a specific host would prefer a mate from the same host plant. Trial 'b' tested for female receptivity as a factor in mating decisions (in no choice assays) by providing the insects that did not mate with a choice. This combination acted as a control to determine whether a female that did not mate actually would if the proper male was present.

STATISTICAL ANALYSIS

Feeding trials

Tests were conducted to determine whether individuals collected from *Gutierrezia* exhibited different host preferences than did individuals from *Solidago* using chi-square analysis. To assess differences in feeding preferences based on host plant affiliations, we grouped and analyzed *Gutierrezia* and *Solidago* populations irrespective of the geographical location from which they were collected. Populations from allopatric and syntopic areas were analyzed separately to detect the presence of differences in host preference between populations. Tests were also conducted to determine

whether the strength of host preferences differed between populations in allopatry versus syntopy using logistic regression by testing for an interaction between host plant use and geography. The presence of a significant interaction term indicates that asymmetries in the strength of feeding preferences in allopatry and syntopy are present. We also tested for character displacement in feeding preference in the zone of syntopy using the procedure of Nosil, Sandoval & Crespi (2006) to estimate divergence for populations. Divergence for pairs of allopatric and pairs of syntopic populations was calculated as % individuals feeding on *Gutierrezia* preferring *Gutierrezia* – % individuals from *Solidago* preferring *Gutierrezia*. All analyses were performed using *R* software, version 2.9.0 (R Development Core Team, 2009).

Near infrared spectroscopy: data validation and analysis

NIR spectral data were validated and analyzed using partial least squares (PLS) regression analysis with GRAMS32 software (Thermo Galactic Industries, 1996) for two-way comparisons between host plant use. Spectral scores were regressed against the host plant group in the PLS regression. One *Gutierrezia* population from Sherman and one *Solidago* population from Marysville were used to develop the initial model, and the remaining six population samples were then used to validate the resulting model. For validation, populations from Sherman and Marysville were treated as known samples, and all samples were subjected to a cut-off 'rejection threshold' of 1.5. Individuals collected on *Gutierrezia* with a partial coefficient > 1.5 or individuals collected on *Solidago* with values < 1.5 were considered misclassified. Wavelengths important in differentiating potential host races were determined using PLS regression coefficient values. The accuracy of classification was interpreted from the percent correct classification, the coefficient of determination, and the standard error of cross validation. Three different sets of PLS regression analyses were performed: (1) All *Gutierrezia* versus all *Solidago* populations: G1 + G2 + G3 + G4 versus S1 + S2 + S3 + S4; (2) *Gutierrezia* versus *Solidago* populations outside the zone of syntopy: G1 + G2 versus S1 + S2; and (3) *Gutierrezia* versus *Solidago* populations within the zone of syntopy: G3 + G4 versus S3 + S4. Analyses were performed for three different wavelengths to identify regions of spectrum most useful in classification: (a) 450–2250 nm; (b) 750–2250 nm; and (c) 450–750 nm.

Mating trials

We calculated a reproductive isolation index I_{PSI} (Rolán-Alvarez & Caballero, 2000), which captures

the intensity of mating isolation between the host plant groups. The significance value and SDs for this index were estimated by resampling the data 10 000 times. Copulation frequencies were analyzed using logistic regression in a model that calculated the probability of copulation based on host species of the male, host species of the female, and an interaction term. A significant interaction between the male and female host indicates that reproductive isolation among the two host forms is present. The significance of the model was evaluated using Akaike information criterion (AIC) (Burnham & Anderson, 1998), which selects for the most likely model at the same time as minimizing the number of assumptions. For the different host combinations, we tested whether reinforcement occurred, in a model where copulation frequencies of males from eight populations were correlated with the male host, female host, and an allopatric term (i.e. the allopatric term was used to test whether females were from an allopatric or syntopic population). All analyses were performed with a reduced regression model (*R* software, version 2.9.0; R Development Core Team, 2009) using backward elimination until the best model with the lowest AIC value was attained. Mean mating probabilities and 95% confidence intervals of different-host and same-host crosses were estimated by logistic analysis of variance using the GENMOD procedure in SAS, version 9.1.3 (SAS Institute, 1990.).

RESULTS

HOST PLANT PREFERENCES

Populations of *H. viridis* collected from *Gutierrezia* and *Solidago* showed significant host preferences, where both groups preferred the primary host from which they were collected ($P < 0.001$; Table 1). Of the insects collected from *Gutierrezia* and *Solidago*, 96% and 84% fed on *Gutierrezia* and *Solidago*, respectively (Fig. 1). We found a significant preference for the home host from which the population was collected in all eight populations (Table 1; all $P < 0.01$).

We analyzed allopatric and syntopic populations separately to determine whether feeding preferences were stronger in syntopic populations and whether character displacement occurred in the zone of syntopy as predicted by the hypothesis of reinforcement. In both allopatry and syntopy, individuals that fed on *Gutierrezia* showed a higher preference for *Gutierrezia* than did individuals that fed on allopatric *Solidago* and *vice versa* ($P < 0.001$; Table 1).

Logistic regression analysis did not reveal any significant interactions between host use \times geography (AIC = 180; $P = 0.47$), indicating that host preference within a plant host group did not differ significantly between individuals from allopatric versus syntopic geographic regions. Tests for character displacement revealed that divergence was lower between syntopic

Table 1. Significance of feeding choice trials between different comparisons of original host plants

	Experiment	Gutierrezia (Solidago)	Solidago (Gutierrezia)	Test statistic χ^2	d.f.	<i>P</i>
A						
1	<i>Gutierrezia</i>	163 (6)		145.85	1	< 0.0001
2	<i>Solidago</i>		110 (20)	64.19	1	< 0.0001
B						
3	Allopatry: Gut versus Sol	63 (3)	57 (5)	94.56	1	< 0.0001
4	Syntopy: Gut versus Sol	100 (3)	53 (15)	101.31	1	< 0.0001
C						
5	Ellis Gut	51	1	48.07	1	< 0.0001
6	Russell Gut	49	2	43.31	1	< 0.0001
7	Hamilton Gut	28	2	22.53	1	< 0.0001
8	Sherman Gut	35	1	32.11	1	< 0.0001
9	Ellis Sol	9	22	5.45	1	0.01
10	Russell Sol	6	31	16.89	1	< 0.0001
11	Konza Sol	2	30	24.50	1	< 0.0001
12	Marysville Sol	3	27	19.20	1	< 0.0001

(A) Chi-squared test results of feeding preference divergence between individuals of *Gutierrezia* and *Solidago* from all populations combined. (B) Divergence in feeding preference among individuals of allopatric and syntopic populations. In (A) and (B), the number of insects feeding on the alternate host is indicated in parentheses. (C). Feeding preference divergence among individuals of eight populations in study zone in Kansas.

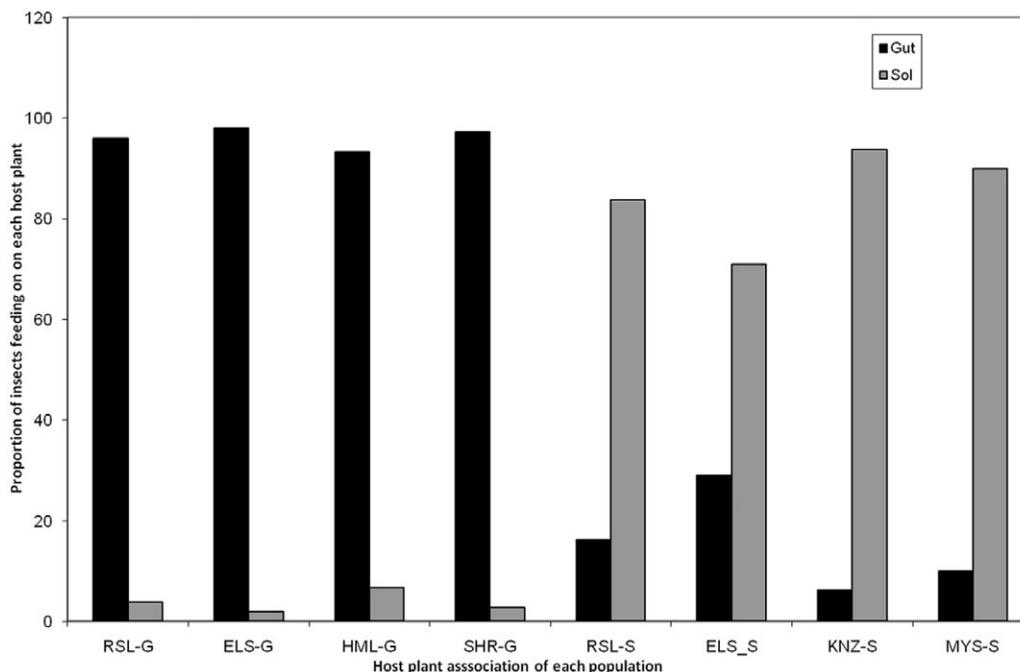


Figure 1. Proportion of individuals feeding on *Gutierrezia* and *Solidago* in each population of *Hesperotettix viridis* in Kansas. ELS, Ellis; RSL, Russell; HML, Hamilton; SHR, Sherman; KNZ, Konza; MYS, Marysville; G, *Gutierrezia*; S, *Solidago*.

population pairs than allopatric populations, and such a trend is opposite to that expected if character displacement in feeding behaviour occurred. The mean of differences in syntopy was 74.5% (SD = 7.7), and 93.2% (SD = 5.3) for the two pairs of allopatric populations ($P = 0.03$).

CUTICULAR NIR PROFILES

Cross-validation results of NIR spectra reveal that 96.4% of individuals were correctly classified into the correct host plant cluster in comparisons of all individuals collected from *Gutierrezia* versus all individuals from *Solidago*. In comparisons among populations outside the zone of syntopy, 99.4% of all individuals were correctly differentiated into respective clusters of those that fed on *Gutierrezia* versus *Solidago*. Within the zone of syntopy, 96.7% of individuals were correctly assigned in comparisons of those that fed on *Gutierrezia* versus *Solidago*. The coefficient of determination (R^2) for the calibration model of all *Gutierrezia* versus all *Solidago* was 0.80, with a value of 0.84 for comparisons of *Gutierrezia* versus *Solidago* outside the zone of syntopy, and 0.81 for comparisons of *Gutierrezia* versus *Solidago* within the zone of syntopy. Within and outside the zone of syntopy, similar patterns of classification success and strength of correlation were

observed (Table 2). Eight wavelengths were useful in identifying *H. viridis* populations in the three comparisons among models. Three wavelengths of 510, 690, and 722 nm were in the visible region of the spectrum and 800, 1050, 1360, 1820, and 1900 nm were in the NIR region (Table 2). Of all the wavelengths useful in discriminating among *H. viridis* populations according to host plant use and based on percent correct classification, the visible region of the spectrum representing colour differences was most important. Other important regions useful in identification represent the CH₃ combination overtone (1360 nm), O-H stretch/C-O stretch second overtone combination corresponding to cellulose in plants (1820 nm), and C=O stretch second overtone corresponding to (–CO₂H) carboxylic group (1900 nm) (Shenk, Workman & Westerhaus, 1992). The average spectra of all *Gutierrezia* and all *Solidago* populations of *H. viridis* are shown in Figure 2.

Calibrations at different wavelengths (450–750, 750–2250, and 450–2250 nm) using the Sherman (G1) Marysville (S1) models permitted us to infer the relative importance of different regions of the spectrum in distinguishing between the host races. The visual region of the spectrum was the most important region enabling identification between the host races with 97.9% and 98.1% correct classification

Table 2. Results of cross validation and partial least square regression analysis among *Hesperotettix viridis* populations

Comparisons/model	Number of factors (required for model calibration)	R^2	SECV	Percent correct classification		
				<i>Gutierrezia</i> sp. = 1	<i>Solidago</i> sp. = 2	Weighted
All Gut versus All Sol G1+G2+ G3+G4 versus S1+S2+S3+S4	8	0.80	0.22	97.9	95.2	96.4
Outside zone of Syntopy Gut versus Sol G1+G2 versus S1+S2	8	0.84	0.20	100	98.6	99.4
Zone of Syntopy Gut versus Sol G3+G4 versus S3+S4	8	0.81	0.21	96.8	98.1	96.7
Gut versus Gut G1+G2 versus G3+G4	8	0.36	0.40	85.0	78.7	81.9
Sol versus Sol S1+S2 versus S3+S4	8	0.20	0.45	80.3	64.2	72.6

R^2 , coefficient of determination (% of variance explained by model); SECV, standard error of cross validation.

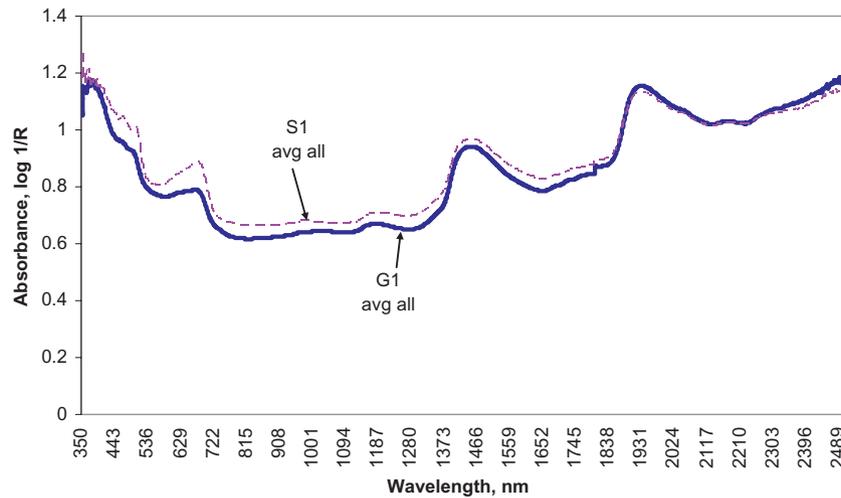


Figure 2. Average near-infrared (NIR) spectra of all *Gutierrezia* and all *Solidago* populations of *Hesperotettix viridis*: G1, *Gutierrezia* sp.; S1, *Solidago* sp.

into *Gutierrezia* and *Solidago* clusters. The 750–2500-nm wavelength region could correctly identify the individuals of *Gutierrezia* and *Solidago* populations with 81.4% and 98.0% accuracy (Table 3). When the entire region of the spectrum was considered (450–2500 nm), accuracy was 95.2% and 98.1% for *Gutierrezia* and *Solidago* individuals, respectively.

MATING TESTS

No-choice mating trials revealed strong host-based sexual isolation between the two host plant groups. Irrespective of the geographic location (allopatric or syntopic), insects in trials from a host plant population always mated at higher proportions with individuals from the same host plant than in trials where a mate from the other host plant group was provided

(Fig. 3; Tables 4, 5). The mean \pm SD duration of copulation among different host plant mating pairs (4.46 ± 2.56 h) did not vary significantly from the same host plant mating pairs (4.29 ± 2.05 h). The mean \pm SD magnitude of sexual isolation varied from 0.46 ± 0.20 in Ellis *Gut* versus *Sol* (syntopic) to 0.89 ± 0.11 in Marysville *Sol* versus Sherman *Gut* (allopatric) comparisons, with an average value of 0.75 ± 0.13 across all different host comparisons (Table 4). Within comparisons of the same host, sexual isolation index values were very low (< 0.036 in all comparisons), indicating little to no sexual isolation among individuals from the same hosts. A significant interaction between male-host and female-host was observed in the logistic regression model, indicating strong host-associated divergence in mating frequencies (AIC value = 144; d.f. = 1; $P < 0.0001$). A model that tested for copulation

Table 3. A posteriori predictions of host plant origin using near infra-red spectra of cuticle at different wavelengths using G1 (Sherman) and S1 (Marysville) calibration models (see text) to predict G2 (Hamilton), G3 (Ellis), G4 (Russell), S2 (Konza), S3 (Ellis), and S4 (Russell)

Population location	Host plant	Host plant-based prediction percent correct classification		
		450–2250 nm	450–750 nm	750–2250 nm
Hamilton (G2)	<i>Gutierrezia</i> sp.	98.0	100.0	82.4
Ellis* (G3)	<i>Gutierrezia</i> sp.	93.2	95.5	81.8
Russell* (G4)	<i>Gutierrezia</i> sp.	94.0	98.0	80.0
Combined G2+G3+G4	<i>Gutierrezia</i> sp.	95.2	97.9	81.4
Konza (S2)	<i>Solidago</i> sp.	100.0	100.0	96.8
Ellis* (S3)	<i>Solidago</i> sp.	100.0	100.0	100.0
Russell* (S4)	<i>Solidago</i> sp.	95.0	95.0	97.5
Combined S2+S3+S4	<i>Solidago</i> sp.	98.1	98.1	98.0

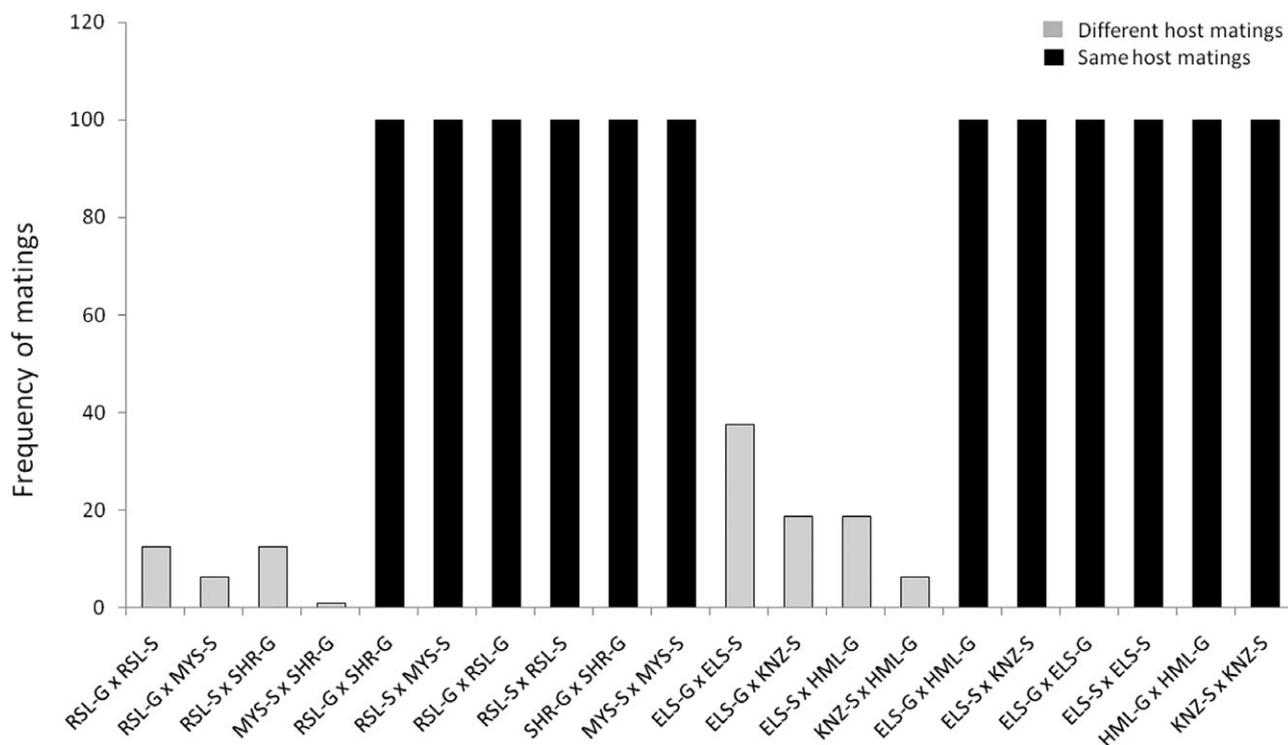


Figure 3. Frequency of matings (no-choice trials) in different host and same host trials. ELS, Ellis; RSL, Russell; HML, Hamilton; SHR, Sherman; KNZ, Konza; MYS, Marysville; G, *Gutierrezia*; S, *Solidago*.

frequencies of males with females (from either allopatry or syntopy) revealed a negative correlation between mating frequencies and geographic distance. (AIC value = 99.11; $P = 0.041$).

When insects mated with individuals from different hosts in no choice trials, we submitted them to a mate choice trial ($N = 18$). Mating occurred with an

individual from the same host plant in all but one trial. In this one trial, the female was not receptive to any male. Similarly, in trials where insects (males or females) did not mate originally when presented with an individual from the alternate host plant group, individuals mated 100% of the time when a choice from the same host was provided. When combined,

Table 4. Magnitude of sexual isolation (I_{PSI}) among populations of *Hesperotettix viridis*

	Host comparison	I_{PSI} sexual isolation index.	<i>P</i>
1	RSL Gut versus RSL Sol	0.78 ± 0.15	0.0004
2	RSL Gut versus MYS Sol	0.84 ± 0.12	0.0002
3	RSL Sol versus SHR Gut	0.75 ± 0.15	0.0006
4	MYS Sol versus SHR Gut	0.89 ± 0.11	0.0002
5	RSL Gut versus SHR Gut	-0.040 ± 0.19	0.86
6	RSL Sol versus MYS Sol	-0.040 ± 0.18	0.85
7	RSL Gut versus RSL Gut	0.0006 ± 0.18	0.97
8	RSL Sol versus RSL Sol	0.0006 ± 0.18	0.97
9	SHR Gut versus SHR Gut	0.0360 ± 0.18	0.85
10	MYS Sol versus MYS Sol	0.0006 ± 0.18	0.97
11	ELS Gut versus ELS Sol	0.46 ± 0.20	0.03
12	ELS Gut versus KNZ Sol	0.76 ± 0.14	0.0006
13	ELS Sol versus HML Gut	0.68 ± 0.15	0.0008
14	KNZ Sol versus HML Gut	0.84 ± 0.12	0.0002
15	ELS Gut versus HML Gut	-0.001 ± 0.18	0.95
16	ELS SOL versus KNZ Sol	0.0020 ± 0.18	0.98
17	ELS Gut versus ELS Gut	0.0006 ± 0.18	0.97
18	ELS Sol versus ELS Sol	0.0360 ± 0.18	0.85
19	HML Gut versus HML Gut	0.0006 ± 0.18	0.97
20	KNZ Sol versus KNZ Sol	0.0006 ± 0.18	0.97

The I_{PSI} index is calculated according to Rolán-Alvarez & Caballero (2000), with a maximum value of isolation of 1. Sites: Ellis (ELS), Russell (RSL), Hamilton (HML), Sherman (SHR), Konza (KNZ), and Marysville (MYS). Host plants: *Gutierrezia* (GUT) and *Solidago* (SOL). Different host mating trials are indicated by shading (total of four trials).

Table 5. 95% confidence intervals for mating probabilities estimated using Logistic regression

	Lower	Mean	Upper
<i>Gutierrezia</i> versus <i>Gutierrezia</i>	0.91	0.97	0.99
<i>Gutierrezia</i> versus <i>Solidago</i>	0.09	0.14	0.21
<i>Solidago</i> versus <i>Solidago</i>	0.93	0.99	0.99

these results suggest strong assortative mating based on host plant affinity.

DISCUSSION

Early stages of speciation are characterized by the initiation of genetic differentiation in response to combined effects of reduced gene flow, genetic drift or natural selection, as may occur when insect herbivores shift to different host species. In the present study, host-associated feeding divergence, assortative mating associated with host plant use, and divergence in cuticular characteristics was observed in *H. viridis* feeding on *Solidago* and *Gutierrezia* hosts. We found no evidence for reinforcement in *H. viridis* in the areas of host plant syntopy.

FEEDING PREFERENCE AND CHARACTER DISPLACEMENT

In insect herbivores, host switching is often constrained by performance limitations on other hosts, which in turn reinforces the evolution of host specialization (Joshi & Thompson, 1995; Berenbaum & Zangerl, 1998), and strong selection against host switching could lead to the evolution of preference for alternate hosts (Nosil *et al.*, 2006). We observed strong feeding preferences for 'home' hosts in all of the Kansas populations studied. Divergence in feeding preference was not significantly different between syntopic populations versus allopatric populations. This result contrasts to that expected if character displacement is occurring in the zone of syntopy, but perhaps is not unexpected for syntopic populations whose microniches do not overlap. The latter is more probable because *H. viridis* individuals are not very mobile and prefer to live in close vicinity to their host plant.

PHENOTYPIC DIVERGENCE IN COLOUR AND CUTICULAR CHARACTERISTICS

The present study revealed significant host-based phenotypic differences in colour and cuticular compo-

sition among *H. viridis* populations in association with host plant use. Colour and cuticular variation observed in the NIR study in the two host forms may aid *H. viridis* with respect to mate recognition and lead to further divergence of mate preferences. Alternatively, the phenotypic differentiation could also be a by-product of host-associated selection and divergence. No character displacement in cuticular characteristics was observed because lower differentiation was observed in the zone of syntopy than in allopatry.

Colour or cuticular hydrocarbons contribute to mate selection in some orthopterans (Tregenza & Wedell, 1997), and mediate kin selection in others (Simmons, 1990). Rapid evolution for changes in cuticular lipid composition among species is known from speciation in some crickets (Mullen *et al.*, 2006). Assortative mating may also result from variation in cuticular hydrocarbons, as observed in a hybrid zone between subspecies of the meadow grasshopper *Chorthippus parallelus* (Neems & Butlin, 1994; Tregenza *et al.*, 2000). Sexual isolation is key to understanding speciation and such studies indicate the roles contributed by cuticular attributes to reproductive isolation. Using all the wavelengths in the visual (colour) and NIR region gave the most accurate identification of the host race. The strength of our coefficient of determination and classification accuracy was comparable to that of the species differences observed between tobacco budworm and corn earworm (Jia *et al.*, 2007). Wavelengths of 450–700, 900–1400, and 1500–1700 nm have previously been shown to be useful in differentiating insect species (Dowell *et al.*, 1999). The present study identified two additional wavelengths of 1820 and 1900 nm that could be used in differentiating *H. viridis* variants.

Cuticular lipids have been implicated in the mate recognition process of several insect species (Tregenza *et al.*, 2000; Dapporto, 2007). The differentiation in the NIR spectra observed for individuals feeding on *Gutierrezia* and *Solidago* suggests the possibility of host-based variation in some chemical components of the cuticle in the two insect forms, which are not yet specified. Hydrocarbons make up major components of cuticular lipids (approximately 32% of lipids in rice weevils: Baker *et al.*, 1984; 48–58% in Mormon cricket: Baker *et al.*, 1960). The 1360-nm wavelength corresponds to CH₃, an important chemical moiety of components that make up the epicuticular lipids in insects (Dowell *et al.*, 1999). The 1820-nm wavelength corresponds to the structural polysaccharide cellulose, which makes up the cell wall in plants (Shenk *et al.*, 1992). Because chitin is the corresponding exoskeleton polysaccharide in insects, we hypothesize that the 1820-nm wavelength may represent variation in chitin composition in *H. viridis*. A wavelength of 1900 nm corresponds to –CO₂H (carboxylic group), a

component of the hydrocarbon biosynthesis pathway in insects (Major & Blomquist, 1978).

MATE SELECTION AND REINFORCEMENT IN THE ZONE OF SYNTOPY

Reinforcement reduces gene flow and hybridization (Nosil, 2005), contributing to population divergence. We found a strong positive relationship between feeding preference on one or the other host and the degree of sexual isolation, yet the magnitudes of divergence were not greater in areas where both host plants were found together. These results are not consistent with the reinforcement hypothesis. Selection for host use may have increased the amount of divergence in allopatric populations because of the absence of alternate hosts and a lack of gene flow. This conclusion is supported by the lower strength of divergence in the feeding preference in the zone of syntopy. Spatial variation in selection could lead to divergence, the tempo of which depends on a balance between selection and the counteracting effects of gene flow (Slatkin, 1987; Nosil, 2005). Gene flow counteracts population divergence between populations except when *F*₁ individuals experience reduced fitness. Thus, gene flow could either enhance or erode genetic differentiation. Selection against host switching contributes to divergence in syntopy in Kansas even with modest gene flow (Grace, 2009). Our recent genetic studies using mitochondrial and microsatellite DNA markers revealed a genetic component to the host-associated divergence in *H. viridis* populations in Kansas (Grace, 2009). Nucleotide substitutions using mitochondrial DNA suggests that the Kansas population is derived from one lineage that expanded from a single refugium followed by radiation and rapid population expansion. We found strong selection associated with host plant use at a single microsatellite locus, further supporting the notion that host plant use is an important mechanism responsible for structuring *H. viridis* populations in Kansas. The locus under strong selection could be linked to a fitness-related gene that affects performance on different host species. The lack of reinforcement in the zone of syntopy may indicate that either too little time has passed for reinforcement to occur subsequent to divergence into two host forms, or fine-scale niche partitioning is effectively separating populations. A lack of divergence and some gene flow at neutral loci in genetic studies is consistent with the idea that host race formation is incomplete. Strong assortative mating based on host plants, poor performance on alternate host plants, physiological and morphological distinctions among host races, and the genetic structure

underlying divergence suggests that this grasshopper species is only partially flexible in its ability to respond to host plant switches.

CONCLUSIONS

Feeding preference tests, mating trials, and genetic data (Grace, 2009) provide evidence for strong selection and adaptation to different host plants as the evolutionary force behind the observed divergence in reproductive isolation. Because patterns of divergence among the allopatric populations did not vary significantly from that of syntopic areas, nor show isolation by distance, it is unlikely that genetic drift played a major role in the observed pattern. Microhabitat selection by *H. viridis* is also highly restrictive, with nymphal development, growth, feeding, mating, and other activities all taking place on their respective host plants (Parker & Root, 1981; Parker, 1982, 1984). Such a strong association with the host plant and the associated preference is likely to foster segregation conducive to the evolution of reproductive isolation.

It has also been reported that host preference could evolve in parapatry from direct selection on colour patterns, through indirect selection (Nosil *et al.*, 2006) or as a result of natal experience of animals (Davis & Stamps, 2004). However evidence from multiple studies allows this possibility alone to be rejected because we found significant variation among the host forms in colour, cuticular composition, feeding preference, mate choice, and genetic support for divergence. Host shifts that enable feeding divergence and habitat isolation could be the initial barrier to gene flow, followed by pre-mating isolation among the two groups with increased sexual isolation. In the present study, host-associated variants of *H. viridis* were identified with high accuracy, suggesting that phenotypic differentiation associated with host plant preferences has occurred. Whether divergence in phenotypic traits contributed to the evolution of mating divergence or evolved as a result of feeding divergence on alternate hosts in response to other selective pressures is unclear. Strong selection could act on all these forces in an additive way to further divergence. Observed host-based differences in phenotypic characters, feeding divergence, and sexual isolations all suggest populations in an intermediate stage of diversification and speciation. Additional information from microsatellite genetic data helps us to validate this prediction of diversification. *Hesperotettix viridis* populations thus represent two host-related forms as expected in incipient speciation. Additional experiments where individuals from both host plants grown in a common environment are subjected to host choice and mate choice trials, as well as an assessment of

phenotypic divergence, will be very useful in confirming the genetic basis of the observed divergence.

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SUPPORTING INFORMATION

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

Table S1. Sampling locations and host plant association of *Hesperotettix viridis* populations used in the near-infrared spectroscopy study.

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