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The evolution of host preference in allopatric vs. parapatric populations of *Timema cristinae* walking-sticks

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Abstract

Divergent habitat preferences can contribute to speciation, as has been observed for host-plant preferences in phytophagous insects. Geographic variation in host preference can provide insight into the causes of preference evolution. For example, selection against maladaptive host-switching occurs only when multiple hosts are available in the local environment and can result in greater divergence in regions with multiple vs. a single host. Conversely, costs of finding a suitable host can select for preference even in populations using a single host. Some populations of *Timema cristinae* occur in regions with only one host-plant species present (in allopatry, surrounded by unsuitable hosts) whereas others occur in regions with two host-plant species adjacent to one another (in parapatry). Here, we use host choice and reciprocal-rearing experiments to document genetic divergence in host preference among 33 populations of *T. cristinae*. Populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did populations feeding on *Adenostoma*. Both allopatric and parapatric pairs of populations using the different hosts exhibited divergent host preferences, but the degree of divergence tended to be greater between allopatric pairs. Thus, gene flow between parapatric populations apparently constrains divergence. Host preferences led to levels of premating isolation between populations using alternate hosts that were comparable in magnitude to previously documented premating isolation caused by natural and sexual selection against migrants between hosts. Our findings demonstrate how gene flow and different forms of selection interact to determine the magnitude of reproductive isolation observed in nature.

Introduction

Natural selection plays a role in speciation when it causes the evolution of reproductive isolation (RI) (Funk, 1998; Schluter, 2000; Kirkpatrick & Ravigne, 2002; Coyne & Orr, 2004). Divergent habitat preferences cause premating isolation when they reduce encounters, and thus matings, between individuals from different populations (i.e. 'habitat isolation', Tavormina, 1982; Rice & Salt, 1988; Stanhope *et al.*, 1992; Craig *et al.*, 1993; Duffy, 1996; Via, 1999; Coyne & Orr, 2004). Host-plant preferences in phytophagous insects can cause habitat isolation because many such insects feed, mate and oviposit exclusively on their hosts (Hawthorne & Via, 2001; Funk *et al.*, 2002). Although host preferences are common in phytophagous insects, geographic variation in preference and its causes is rarely described (but see Jaenike & Grimaldi, 1983; Forister, 2004). Also, some studies testing for divergent preferences do not detect them (e.g. Jiggins *et al.*, 1997; Poore & Steinberg, 2001). Thus, the factors both driving and constraining the evolution of host preference require further study (Jaenike & Holt, 1991; Carriere, 1998).

Here, we examine the role of three evolutionary processes in structuring geographic variation in host preference among populations of walking-stick insects: (i) selection for reduced search costs and efficient host

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Table 1 Hypotheses and predictions for the evolution of habitat preferences.

Evolutionary mechanism	Geographic context	Predictions regarding the geographic variation
Selection to reduce search costs and to increase efficiency; can act directly on preference loci	Can act in populations where a single or multiple habitats are available	Divergence between populations using alternate habitats, even when they each use a single (but different) habitat
Selection against habitat-switching; often acts indirectly on preference loci via their genetic association with fitness loci	Acts only in populations where multiple habitats exist (i.e. where there is the opportunity for habitat-switching)	Greater divergence between parapatric/sympatric populations than between allopatric populations
Gene flow	Acts when gene flow can occur between populations using alternate habitats, most likely in parapatry/sympatry	Greater divergence between allopatric populations than between parapatric/sympatric populations
Balance between the selection and gene flow	N/A	Variable, depending on the relative strength of different forms of selection and levels of gene flow

finding, (ii) selection against maladaptive host-switching, and (iii) gene flow between populations. We refer to host-plant preferences of herbivores throughout but stress that the hypotheses and implications apply to the habitat preferences of many organisms.

Host preferences can diverge both with and without selection against switching between different, utilized hosts (we use the term ‘utilized’ to refer to the host species that an insect species uses; other host species that the insect species cannot or does not use may exist in the environment as well). There is no selection against switching between utilized hosts when only one host is utilized in the local environment. Under this scenario, search and efficiency costs can favour the increased preference for the single, utilized host because individuals without strong preferences accrue lower fitness, but for reasons other than switching to an alternate host (Jaenike & Holt 1991; Bernays & Wcislo, 1994; Janz & Nylin, 1997; Carriere, 1998; Bernays & Funk, 1999). For example, such individuals might take longer to locate or to decide whether to feed on the utilized host, thereby wasting time and energy while increasing predation risk. Alternatively, individuals may suffer low fitness because they attempt to use a ‘nonutilizable’ host. When preference evolution is driven by such selection, populations in habitats where only a single host is utilized still evolve preference for that host.

When switching between utilized hosts is maladaptive (i.e. when local adaptation results in fitness trade-offs between hosts), host preferences can also diverge via selection against the individuals that switch between hosts (Balkau & Feldman, 1973; Kawecki, 2004). Under this scenario, preference for one host is favoured because individuals choosing another host suffer reduced fitness (but see Fry, 1996; Kawecki, 1996, 1997). This form of selection only acts in populations where there is the opportunity for switching between the utilized hosts (i.e. when more than one utilized host is available in the environment) and forms the cornerstone of many verbal and mathematical models of sympatric speciation (Bush, 1969; Johnson *et al.*, 1996; Berlocher & Feder, 2002; Dres & Mallet, 2002; Kawecki, 2004 for review). Although selection in this scenario actively favours the reduced host-switching, it often acts on host preference loci indirectly via their genetic association with loci conferring host-specific fitness (Kirkpatrick & Barton, 1997). One possible outcome of this process is greater preference divergence in geographic regions where multiple hosts are utilized (in sympatry or parapatry) than between geographically isolated populations that use a single, yet different, host (allopatry). We will refer to this pattern as ‘character displacement’ of host preference.

In contrast to the forms of selection described above, gene flow often erodes population differentiation (Slatkin, 1987; Hendry *et al.*, 2001, 2002; Hendry & Taylor, 2004). When gene flow constrains divergence, populations exchanging genes in sympatry or parapatry exhibit weaker divergence than do geographically isolated, allopatric populations. The scenarios outlined above consider each of the evolutionary forces in isolation, but evolution in nature will reflect a balance between these different processes (Table 1).

In this study, we examine the host preferences of *Timema* walking-sticks feeding on one of two distinct host-plant species (*Ceanothus spinosus* or *Adenostoma fasciculatum*) under allopatry (one host available in the local environment) and parapatry (both hosts available in the local environment). The study has four main goals: (i) to test whether host preferences differ between populations using different hosts, (ii) to assess whether population divergence has a genetic basis, (iii) to estimate the importance of host preference relative to other premating barriers, and (iv) to ascertain which evolutionary processes explain the among-population variation in host preference. With respect to evolutionary processes, explicit predictions can be made. Preference evolution driven by search costs predicts divergence even between allopatric populations that use a single (but different) host each. If selection against maladaptive host-switching is important, then ‘character displacement’ of host preference is expected. Finally, if gene flow constrains differentiation, then preference divergence

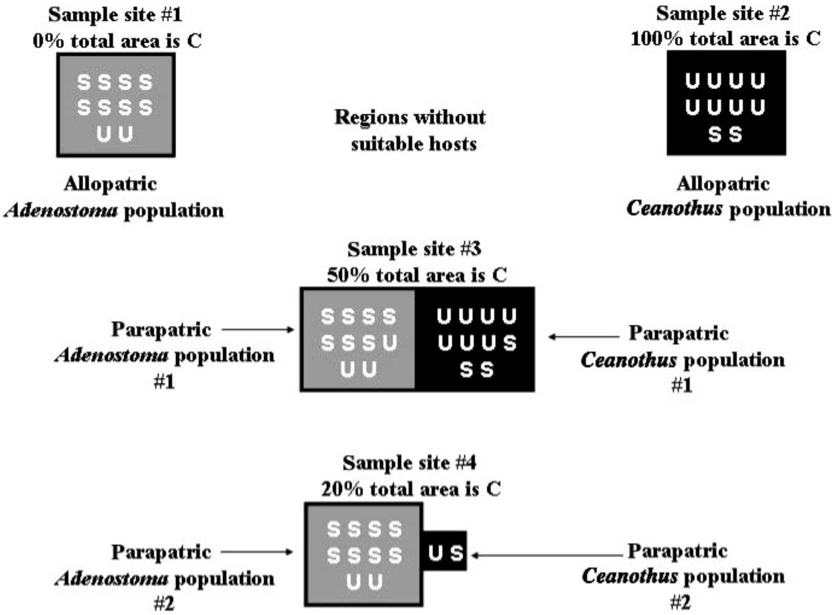

Fig. 1 Schematic representation of the different types of populations examined. A 'sample site' is defined as a contiguous area of one or both hosts that is separated from all other sample sites by regions without suitable hosts. A 'population' of walking-sticks is defined as all the insects captured within a homogeneous patch of a single host-plant species (light boxes = *Adenostoma* populations; dark boxes = *Ceanothus* populations). Thus, parapatric populations have an adjacent population that uses the alternate host whereas allopatric populations do not (therefore six populations are depicted above). Also, shown is the proportion of the total area of a sample site that is occupied by *Ceanothus* (C). Letters within the boxes denote striped (S) vs. unstriped (U) colour-pattern morphs within populations. The current study focuses on divergence in mean host preference between populations, whereas a related study examined genetic covariance between colour-pattern and host preference within populations.

will be weaker when migration between utilizable hosts occurs (in parapatry) than when it does not (in allopatry). In addition, we provide an estimate of total premating isolation between the host-associated forms of *Timema cristinae* by combining the results on host preference with previous estimates of premating isolation caused by natural and sexual selection against the between-host migrants (i.e. 'immigrant inviability' and 'sexual isolation' respectively; Nosil *et al.*, 2002, 2003; Nosil, 2004; Nosil *et al.*, 2005). Collectively, the results help explain the processes driving and constraining the evolution of reproductive barriers.

Study system

Timema walking-sticks are wingless insects inhabiting the chaparral of Southwestern North America (Vickery, 1993; Crespi & Sandoval, 2000). Individuals feed and mate exclusively on the hosts upon which they rest and thus host preference can result in premating isolation. Patches of the two host species used by *T. cristinae* are usually distributed in parapatric patches of varying size. However, some host patches are geographically separated from all others by regions lacking suitable hosts (Fig. 1). Each 'sample site' is contiguous area of one or both hosts that is separated from all other sample sites by regions without suitable hosts. We focus on divergence between populations, where a 'population' of walking-sticks is defined as all of the insects collected within a homogenous patch of a single host-plant species (as in Nosil *et al.*, 2002, 2003). Thus, 'Parapatric' insect populations are in contact with a population of insects adapted to the alternative host (i.e. they have a 'neighbouring', adjacent population using the alternative host), whereas 'allopatric' populations are separated from all other populations adapted to the alternative host by distances >50 times the 12-m per-generation gene flow distance (Sandoval, 1993). Sample sites with both hosts were chosen such that there was only one population on each host species.

Previously published studies of *T. cristinae* examined a number of factors other than host preference. These studies documented adaptive morphological divergence (Sandoval, 1994a; Nosil & Crespi, 2004) and RI between populations using different hosts caused by immigrant inviability and sexual isolation (Nosil *et al.*, 2002, 2003; Nosil, 2004; Nosil *et al.*, 2005). We have now examined the host preference in this system in two distinct contexts. A related study has shown that the genetic covariance between colour-pattern and host preference within populations is greater in parapatric populations (Nosil *et al.*, in press; see Discussion for summary).

Divergence in mean host preference between populations is the topic of the current study and has not been examined previously.

The conditions for the evolutionary processes outlined above to contribute to preference evolution are met. Firstly, selection for specialization because of search and efficiency costs can occur. For example, *Timema* are heavily preyed upon by visual predators (birds, lizards; Sandoval, 1994a,b; Nosil, 2004) and time spent searching for or deciding whether to rest upon a host could increase predation risk. Secondly, selection against maladaptive host-switching can occur in parapatric populations. Each of two main colour-pattern morphs in *T. cristinae* has higher survival on the host-plant on which it is more common, because of differential visual predation (Sandoval, 1994a,b; Nosil, 2004). Thus, divergent selection acts on colour-pattern and, on average, switching hosts result in low survival (Nosil, 2004). Selection can act indirectly on host preference loci within parapatric populations via the positive genetic covariance between colour-pattern and host preference (Nosil *et al.*, in press). Finally, both morphological and mitochondrial DNA sequence divergence is consistently lower between adjacent, parapatric pairs of populations than between geographically separated pairs of populations. This pattern indicates gene flow between parapatric populations (Sandoval, 1994a; Nosil *et al.*, 2003; Nosil & Crespi, 2004).

Populations of *T. cristinae* exist in a geographic mosaic. It is unlikely that every population represents an entirely independent evolutionary replicate because evolution within each population may have a different starting point, depending on colonization history (i.e. on the preference of ancestral populations). Current day preference represents a combination of the retention of ancestral preference and evolution towards or away from it. We do not claim that differences between populations represent 'divergence' in the sense that they evolved totally *in situ*. Rather, we examine the general trends across multiple populations on different hosts and try to ascertain what processes account for the variation in host preference. We focus throughout on comparing data combined from multiple populations that are similar in host use or geography to data combined from multiple populations that are dissimilar, yielding large sample sizes for most of our analyses. We note the few cases where we do examine the individual populations are warranted, because there is evidence for independent evolution within populations occupying different geographic regions (Nosil *et al.*, 2002, 2003).

Materials and methods

Field collecting and insect maintenance

Timema cristinae were collected from 33 populations in the Santa Ynez Mountains, California between January and June in 1992, 1996, 2001–2004 using sweep nets. Other species of *Timema* do not occur in sympatry with *T. cristinae*. Walking-sticks were maintained in glass jars at the University of California at Santa Barbara (20 °C) with 10–15 individuals per jar. Individuals from different populations and the sexes were kept separate. Animals were fed the foliage of *Ceanothus*, except in the case of the reciprocal-rearing experiment (see below). Appendix 1 provides a description of each population and population-specific sample size sizes for each experiment.

Host-preference experiments

Experiment #1 (field-caught individuals, single insect per replicate)

All the experiments were a choice situation because individuals of *T. cristinae* will accept their non-native host if given no choice and can be reared successfully on either host. Host-preference tests were performed using randomly collected insects. Individual walking-sticks (total $n = 1426$) were placed in the bottom of a 500-mL plastic cup (height, 15 cm) with one 12-cm host cutting from each host-plant species in the cup. The bottom end of each host cutting was placed in a plastic aqua-pic filled with water, which held the cutting upright and kept it fresh. The top of each container was covered with wire netting secured by elastic bands. Assays were initiated in the evening and test animals were left in darkness overnight. In the morning, we recorded which host species each individual was resting on. For assays where the test individual did not choose a host (i.e. they were resting on the container, <5% of trials), the container was left overnight until a host was chosen (for up to two nights). Each individual was used only once. All scoring was carried out blind to population of origin by Nosil.

Experiment #2 (field-caught individuals, multiple insects per replicate)

The second experiment simulated a scenario where multiple individuals might simultaneously be picking a host, as might occur in nature. Preference tests were conducted on walking-sticks collected in 1992 and 1996. In 1992, we offered insects one 30-cm high branch of each host species (branches kept 10 cm apart and out of contact in a Styrofoam sheet floating in a container with water, thereby keeping the plant fresh and preventing insects from escaping). Approximately, 10 insects from the same population were placed on the styrofoam, midway between the two branches, and left overnight. The following morning the number of insects on each branch was counted by Sandoval. The choice test was replicated for each population based on the insect availability. Individuals that did not choose a host (<5%) were excluded from the analysis and each insect was used only once. Because of a shortage of insects in 1996, each replicate had only one insect and the procedure was modified. The two branches of the host plants were placed inside of a 0.5-L plastic cup covered with netting. The plants were kept fresh using water-filled aqua-pics. This slight methodological modification in 1996 is very unlikely to affect our conclusions because it involves only four populations and our conclusions were well supported by the other experiments presented in this study (see Appendix 1, for populations affected). Branches were obtained from the same site as the individuals were collected from. Different plant individuals were used for each replicate and the pair of branches within each replicate collected from adjacent plants in the field. Mean preference from each replicate (% of individuals picking *Ceanothus*) was used as a single data point in all statistical analyses.

Experiment #3 (genetic crosses)

The third experiment provides the same general information as the first two, but additionally represents a common-garden experiment. Individuals from within 20 populations were crossed with one another in 2003 and 2004 ('within-population crosses' – both parents always from the same population). All the individuals used in the crosses were sexually immature instars captured in the field that were reared to sexual maturity in the absence of the opposite sex on *Ceanothus* cuttings. A small number of between-population crosses were also conducted (see below). A single virgin male and a single virgin female were housed together in a petri dish until the copulation was observed and then fed *Ceanothus* cuttings until the female died (females lay single eggs daily). The following spring (after the eggs overwintered) offspring were scored for host preference within a few days of emergence using the same protocol as Experiment #1. Each family mean was used as a single data point in statistical analyses.

A portion of these same data (64 of 145 families and 428 of 988 individuals, all from 2003) comes from an experiment designed to measure the genetic covariance between host preference and colour-pattern. In this experiment, nonrandom mating was imposed such that both parents were always the same colour-pattern morph. This does not qualitatively affect our conclusions in any way because the subset of the crosses where parents were mated randomly with respect to colour-pattern includes all 20 populations and yields the same result as the full database (e.g. mean population preference using only the crosses with random mating is highly correlated with mean population preference using full database, $r = 0.71$, $P < 0.001$).

Statistical analyses

All statistical analyses were conducted using SPSS (v. 12) (Chicago, IL, USA). Trends from the three experiments were always in the same direction. In cases where all three experiments did not yield significant results individually, we also report Fisher's combined probability values (Sokal & Rohlf, 1995).

Geography and population divergence in host-plant preferences

We first tested whether individuals derived from populations feeding on *Ceanothus* exhibit different host preferences than individuals derived from populations feeding on *Adenostoma*. For this analysis, we used chi-squared tests (for Experiment #1, as the preference data were categorical) and *t*-tests (for Experiments #2 and #3, as the preference data were continuous). These analyses use all the data and test for divergence independent of geography. If search costs contribute to evolution, host preference should diverge even between individuals from allopatric populations where there is no opportunity for selection against host-switching. To test this prediction, we repeated the chi-squared and *t*-test analyses, but restricted them to individuals from allopatric populations. We then examined whether difference in host preference occurs in parapatry alone by repeating the analyses using only individuals from parapatric populations.

We assessed whether host preference differed between geographic scenarios (allopatry/parapatry) by testing for an interaction between host-plant used and geography in logistic regression (Experiment #1) and ANOVA analyses (Experiments #2 and #3). The interaction terms test for an effect of geography, but do not explicitly examine the direction of differences. Strengthening of preferences in response to selection against maladaptive host-switching is expected to leave two directional patterns: (i) greater preference for the native host in parapatric vs. allopatric populations and (ii) greater divergence in preference between parapatric vs. allopatric pairs of populations using the alternate hosts.

We examined whether preference for the native host (i.e. the host of the population from which an individual is derived) differs for individuals derived from allopatric populations than those derived from parapatric populations (Experiment #1 – chi-squared tests; Experiments #2 and #3 – *t*-tests). To account for asymmetry in the host preference of populations using different hosts and to avoid confounding difference between hosts with variability among populations within hosts, we conducted separate analyses for each host. The tests above do not account for population-specific variation or examine divergence between population pairs *per se*. Thus, we also compared divergence in host preference between parapatric vs. allopatric pairs of populations that use different hosts. In this analysis, pairs of populations, rather than individuals, become the unit of replication and the difference between population pairs is compared between the two geographic comparisons (parapatric vs. allopatric pairs) using a *t*-test. Allopatric populations were paired randomly into different-host pairs but our results are unaffected by alternative pairings because allopatric populations of the same host tend to have similar host preferences. Parapatric populations were always paired with the adjacent population on the alternative host. Each population was used in only a single pairwise comparison.

Selection-gene flow balance

We assessed whether mean trait values for each single population reflect the effects of a balance between selection and gene flow using a quantitative index of this balance. When populations use *Ceanothus* as a host plant, the size of the adjacent population of *Adenostoma* serves as an index of the opportunity for gene flow to erode local adaptation to *Ceanothus* (population sizes inferred from host-plant patch sizes, see below). Conversely, when populations use *Adenostoma* as a host plant, the size of the adjacent population of *Ceanothus* serves as an index of the opportunity for alleles conferring adaptation to *Ceanothus* to be introduced into the population. Thus, for each study population, the value assigned to it simply represents the proportion of the total area (area of the study population plus the area of the adjacent population using the alternate host) occupied by *Ceanothus*. Allopatric populations (which do not have an adjacent population) apparently undergo little or no gene flow (Nosil *et al.*, 2003), and are assigned values of zero (for *Adenostoma* populations) or 100 (for *Ceanothus* populations). Parapatric populations are assigned values between zero and 100, based upon the relative abundance of *Ceanothus*.

Previous work indicates that this index accurately estimates the geographic potential for gene flow as: (i) field sampling has shown that patch size and population size are strongly, positively correlated (Sandoval, 1994a) and (ii) the relative size of the population using the alternative host that is adjacent to a focal population is strongly correlated with the migration rate from the adjacent population into the focal population (Nosil *et al.*, 2003; Nosil & Crespi, 2004; migration estimated from mtDNA sequence data and the coalescent-based methods of Beerli & Felsenstein, 2001). In particular, we refer readers to Nosil & Crespi (2004) for a detailed validation.

Patch areas were calculated from aerial photographs and ground-truthing (as in Sandoval, 1994a,b; Nosil *et al.*, 2003; Nosil & Crespi, 2004). Spearman's rank correlation was used to test whether mean population preference for *Ceanothus* was correlated with the proportion of the total area occupied by *Ceanothus* (i.e. the index of selection/gene flow balance). To avoid conflating differences between hosts with differences among populations within hosts, analyses were run separately for populations using each host species (for Experiments #1 and #3 only, because of lack of replication among populations using the same host in Experiment #2).

Genetic basis of host preference

Four different lines of evidence were used to assess whether population divergence in host preference has a genetic basis. Firstly, congruence in population divergence between the results from field-caught and genetic cross data suggests a genetic basis to population divergence. Secondly, the genetic crosses represent a common-garden experiment such that differences among populations in Experiment #3 are likely to have a genetic basis. Thirdly, for a subset of the populations studied ($n = 6$) in Experiment #1, we raised some of the individuals on their native host and some on the alternative host (from first instar until sexual maturity comprising approximately 4–6 weeks of rearing, Fig. 3 for sample sizes). We used logistic regression analyses to test whether host picked (*Ceanothus* or *Adenostoma*) in these populations was influenced by genotype, rearing environment (host reared on) or a genotype by environment interaction [assessing significance using likelihood ratio tests (LR)]. We conducted two analyses, one using population of origin as the genotype term and one using host of origin as the genotype term. We report the results from a full model that included both factors and the interaction as well as the results from a reduced regression model derived using backward elimination (the reduced model removes all terms for which the significance of $-2 \log$ LR was >0.10 in the full model). Fourthly, some genetic crosses were also conducted between individuals from different populations using alternate hosts ($n = 26$ families). The preference of the F1 'hybrids' emerging from such crosses ($n = 70$ individuals) was assayed using the protocols in Experiment #1. Hybrid preferences were then compared with the preferences of nymphs emerging from within-population crosses (using only the populations for which both within-population and between-population crosses were conducted).

Components of premating isolation

We estimated the total premating isolation caused by the combined effects of host preference, selection against immigrants (immigrant inviability) and divergent mate preferences (sexual isolation), as well as the relative contribution of each of these three individual components to total isolation [see Ramsey *et al.* (2003), for details of the estimation procedure].

Individual components of RI specify the magnitude of RI caused by a given barrier to gene flow when it acts alone. The individual contribution of host preference (RI_h) was estimated as the absolute value of the (% difference between a population pair in mean preference for *Ceanothus*), immigrant inviability (RI_m) was estimated as [1 − (immigrant survival/resident survival)] and the individual contribution of sexual isolation (RI_s) as [1 − (heterotypic mating frequency/homotypic mating

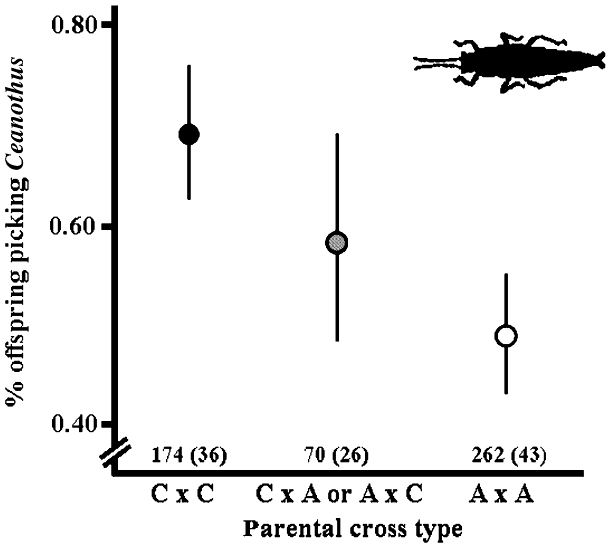

Fig. 2 F1 'hybrids' between host forms show intermediate host preferences. Host preference (mean % picking *Ceanothus* ± 95% CI) of laboratory-emerged nymphs from crosses within vs. between divergent host forms (C × C – both parents from the same population of *Ceanothus;* C × A or A × C – parents from different populations with one parent from each host; A × A – both parents from the same population of *Adenostoma*). Number of individuals for each cross type is shown above the *x*-axis (with number of families in brackets to the right). Individual means are shown but trends with family means are congruent.

frequency)]. Where relevant, the two values from a population pair were always averaged. Total RI is computed as multiplicative function of the individual components at sequential stages in the life history, but a given component of RI can only eliminate gene flow that has not been eliminated by a previous component. Host preference acts before the selection against migrants, which in turn acts before sexual isolation. Thus, the absolute contribution of host preference is ($AC_h = RI_h$), the absolute contribution of selection against migrants is [$AC_m = RI_m \ (1 - AC_h)$], the absolute contribution of sexual isolation is $AC_s = RI_s \ [1 - (AC_h + AC_m)]$ and total isolation is ($AC_h + AC_m + AC_s$). The relative contribution of any component is simply the absolute contribution divided by the total isolation.

We estimated the components of RI between pairs of populations under three major eco-geographical scenarios: (i) allopatric pairs of populations using the same host species, (ii) allopatric pairs of populations using different host species, and (iii) parapatric pairs of populations using the alternate host species. Analyses have already been conducted for immigrant inviability and sexual isolation and estimates of these barriers are taken directly from Nosil (2004). For host preference, we used all the populations for which $n > 5$ for both populations in a population pair in Experiment #1. Allopatric populations were paired randomly into same-host or different-host pairs, using each population in only a single pairwise comparison. Our results are unaffected by the alternative pairings or the use of populations with smaller sample sizes because allopatric populations of the same host tend to have similar host preferences (same-host pair reported – VPC × PE; different-host pairs PR × L and PE × LRN). Parapatric populations were always paired with the adjacent population on the alternative host. When multiple population pairs comprised a single eco-geographic comparison, the overall mean of the different population means was used.

Results

Population divergence in host-plant preferences

Timema cristinae from populations feeding on *Ceanothus* differed significantly in host preference from those feeding on *Adenostoma*. In both experiments using field-caught insects, individuals from populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did individuals from populations feeding on *Adenostoma* (both $P < 0.01$, Table 2). Likewise, laboratory-emerged nymphs whose parents were from populations feeding on *Ceanothus* showed a greater preference for *Ceanothus* than did nymphs whose parents were derived from populations using *Adenostoma* ($P < 0.01$, Table 2).

Divergence in allopatry – 'search costs'

The results above demonstrate that host preference has diverged between the populations using different hosts, but do not test which processes contribute to divergence. To test whether search costs in allopatry contribute to evolution, we repeated the *t*-test and chi-squared analyses reported above, but restricted them to individuals from allopatric populations. Consistent with the divergence of allopatric populations, individuals from allopatric populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did individuals from allopatric populations feeding on *Adenostoma* ($P < 0.01$ in all three experiments, Table 2).

Divergence in parapatry – 'character displacement'

Divergence also occurred in parapatry. Individuals from parapatric populations feeding on *Ceanothus* exhibited a stronger preference for *Ceanothus* than did individuals from parapatric populations feeding on *Adenostoma* ($P < 0.01$, 0.05, 0.52 for Experiments #1–3 respectively, combined probability $P = 0.0011$, Table 2). Host preferences tended to differ between individuals from allopatric vs. parapatric populations, as indicated by host-use × geography interactions in logistic regression ($LR = 18.22$, $P < 0.001$, Experiment #1) and ANOVA analyses ($F_{1,143} = 1.65$, $P = 0.20$; $F_{1,145} = 5.93$,

Table 2 Mean host preference (% picking *Ceanothus*) for individuals from populations of *Timema cristinae* feeding on two different host-plant species (A – *Adenostoma*, C – *Ceanothus*). The data shown are from three independent experiments. Experiment #1 uses field-captured insects with each individual considered a replicate and a chi-squared test was used to determine whether host species picked is dependent on host of origin. Experiment #2 uses field-captured insects with multiple individuals from the same host per replicate. A *t*-test is used to test whether mean preference differs for replicates with individuals from *Ceanothus* vs. *Adenostoma*. Experiment #3 assesses the host preferences of F1 laboratory-emerged nymphs derived from the genetic crosses (both parents from the same host and population). Each family is considered a replicate and a *t*-test on family means is used to test for differences between offspring derived from the parents from populations using *Ceanothus* vs. *Adenostoma*.

Experiment	C populations, mean (SD)	A populations, mean (SD)	Test statistic	d.f.	# Replicates	# Individuals
Pooled						
Experiment #1	90 (30)	72 (45)	79.03***	1	N/A	1426
Experiment #2	75 (36)	56 (35)	2.89**	141	143	710
Experiment #3	67(32)	50 (34)	2.96**	143	145	988
Allopatric only						
Experiment #1	93 (25)	63 (48)	82.30***	1	N/A	615
Experiment #2	78 (15)	30 (12)	5.06***	12	14	117
Experiment #3	72 (25)	40 (35)	4.25***	60	62	480
Parapatric only						
Experiment #1	85 (36)	74 (44)	10.57**	1	N/A	811
Experiment #2	73 (42)	56 (35)	2.18*	127	129	598
Experiment #3	61 (37)	56 (30)	0.65	81	83	508

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

$P < 0.05$, Experiments #2 and #3 respectively; combined probability among experiments $P = 0.001$).

We conducted two explicit tests for character displacement of host preference, both of which yielded no evidence for its occurrence. Firstly, preference for the native host (i.e. the host that the population an individual is derived from uses) tended to be significantly greater for individuals derived from allopatric populations than for individuals derived from parapatric populations, particularly for *Ceanothus* populations (differences were always greater for allopatric populations, even if not statistically so; combined across hosts and experiments $P = 0.0001$; Table 3).

Secondly, comparing population divergence of allopatric vs. parapatric pairs of populations provides the most explicit test for character displacement (where divergence for each individual population pair is calculated as percent of individuals from the *Ceanothus* population preferring *Ceanothus* minus percent of individuals from the *Adenostoma* population preferring *Ceanothus*). Such an analysis of population divergence revealed that, if anything, allopatric pairs show greater mean divergence than parapatric pairs (Experiment #1, mean of differences for six parapatric pairs = 14%, SD = 21, mean of differences for three allopatric pairs = 45%, SD = 43, $t_7 = 1.49$, $P = 0.18$; Experiment #3, mean of differences for four parapatric pairs = −7%, SD = 14, mean of differences for two allopatric pairs = 22%, SD = 10, $t_4 = 2.56$, $P = 0.063$; *t*-tests; combined $P < 0.05$). Thus analyses of mean preference show that parapatric populations do show divergence, but provide no evidence that divergence has been strengthened in parapatry. In fact, it appears that parapatric populations show weaker divergence than the allopatric populations.

Selection-gene flow balance

We tested whether mean preference for each single population could be predicted by host-plant used and the opportunity for homogenizing gene flow. The results provide some support for this hypothesis, dependent on the host species and experiment considered. For populations using *Ceanothus*, mean population preference for *Ceanothus* was significantly correlated with our index of the balance between selection and gene flow for Experiment #1 ($\rho = 0.88$, $P < 0.001$) and was

Table 3 Tests for whether preference for the native host (the host that the population that an individual is derived from uses) differs between individuals from allopatric vs. parapatric populations. Differences between groups were tested using a chi-squared test (Experiment #1) and *t*-tests (Experiments #2 and #3). Table 2 provides mean preferences.

Experiment	Test-statistic	d.f.	*P*-value
Adenostoma populations			
Experiment #1	6.47	1	<0.05
Experiment #2	1.30	102	0.20
Experiment #3	1.91	72	0.06
Ceanothus populations			
Experiment #1	12.76	1	<0.001
Experiment #2	0.35	37	0.73
Experiment #3	1.52	69	0.13

marginally insignificant for Experiment #3 ($\rho = 0.49$, $P = 0.065$). These results held up reasonably well when populations with small sample sizes were excluded ($n > 9$ individuals for Experiment #1 and $n > 4$ families for Experiment #3; $\rho = 0.92$, 0.52, $P < 0.001$, $P = 0.15$, respectively). For populations using *Adenostoma*, the trends were much weaker (using all the populations, $\rho = 0.18$, 0.54, $P = 0.30$, 0.066 for Experiments #1 and #3 respectively, combined $P = 0.098$; excluding small samples, $\rho = 0.24$, 0.48, $P = 0.30$, 0.14, respectively). We return to this variability between hosts in the discussion.

Genetic basis for population divergence

Four different lines of evidence suggest that population divergence in host preference has a strong genetic basis. Firstly, results from both field-caught and laboratory-emerged insects tend to be congruent; in both cases populations from *Ceanothus* exhibited greater preference for that host (Tables 1 and 2; the correlation between population means for Experiments #1 and #2 was $\rho = 0.47$, $P = 0.051$). Secondly, the results from the genetic crosses represent a common-garden experiment and thus differences between the populations likely represent genetic divergence (Table 2). Thirdly, logistic regression analysis of the reciprocal-rearing experiment revealed no evidence that environmental effects (i.e. host species reared upon) influence host preference and strong evidence that genotypic effects do affect host preference (Fig. 3). This strong effect of genotype occurred when the host of origin was used as the genotype term (full model, host of origin $-2LR = 4.10$, d.f. = 1, $P < 0.05$, host reared upon $-2LR = 0.21$, d.f. = 1, $P = 0.65$, interaction $-2LR = 1.13$, d.f. = 1, $P = 0.29$; reduced model, host of origin $-2LR = 12.832$, d.f. = 1, $P < 0.001$, other terms removed) and when population of origin was used as the genotype term (full model, population of origin $-2LR = 8.01$, d.f. = 5, $P = 0.15$, host reared upon $-2LR = 0.61$, d.f. = 1, $P = 0.44$, interaction term $-2LR = 2.86$, d.f. = 5, $P = 0.72$; reduced model, population of origin $-2LR = 16.90$, d.f. = 5, $P < 0.01$, other terms removed). Fourthly, F1 'hybrids' between the host forms exhibit intermediate preferences, indicative of genetic differences between the host forms (Fig. 2).

Components of premating isolation

Total premating isolation is nonexistent for allopatric pairs using the same host, strongest for allopatric pairs using alternate hosts, and intermediate for parapatric pairs using alternate hosts (total isolation = −0.04, 0.67 and 0.51 respectively, Fig. 4). Within each 'eco-geographic' comparison, the individual components of isolation caused by host preference, immigrant inviability and sexual isolation are roughly similar. This similarity

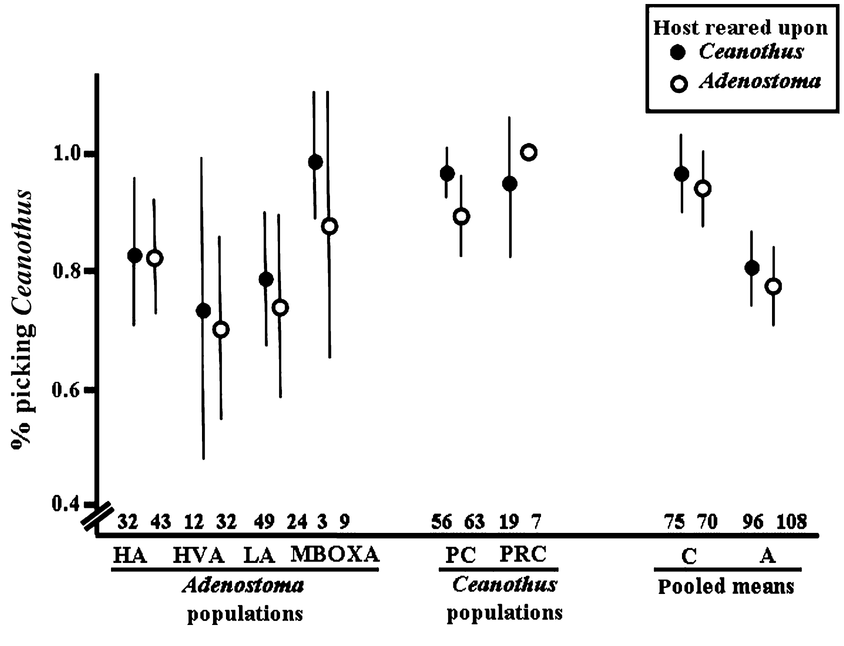

Fig. 3 In a reciprocal-rearing experiment, the effects of rearing were insignificant whereas genotype effects (population and host of origin) were significant (see Results for statistics). Shown is host preference (mean % picking *Ceanothus* ± 95% CI) of field-caught first instars reared until sexual maturity on *Ceanothus* (C) vs. *Adenostoma* (A) (about 4–6 weeks of rearing). Populations adapted to *Adenostoma* are depicted on the left, populations adapted to *Ceanothus* in the centre and pooled means for each host species on the right. Numbers above the *x*-axis refer to the number of individuals tested.

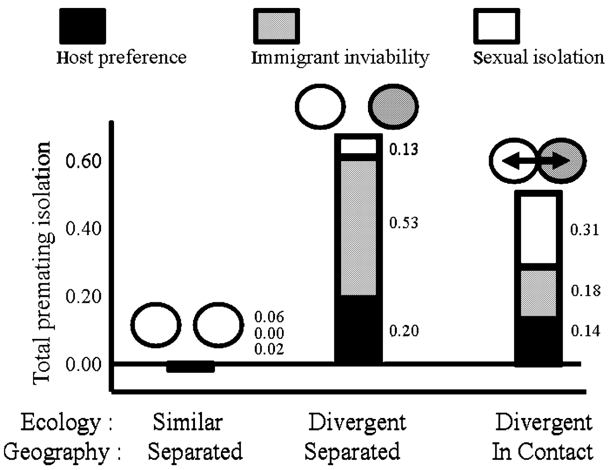

Fig. 4 Components of reproductive isolation (RI) under different ecological and geographical scenarios. Pairs of populations using the same host show very little RI. For populations using different hosts, roughly similar levels of total premating isolation are observed under allopatry and parapatry but arise via different individual components of RI. Shown graphically are the absolute contributions of host preference, immigrant inviability and sexual isolation to total premating isolation. The relative contribution of each component is simply its absolute contribution divided by total isolation. Individual components (strength of the barrier acting in isolation) are labelled to right of the bar for each barrier.

among components is even greater for the absolute contribution to total isolation because host preference acts earliest in the life history. For populations using different hosts, roughly similar levels of total premating isolation are observed under the allopatry and parapatry but arise via different individual components of RI. Specifically, host preference and immigrant inviability contribute strongly under the allopatry whereas sexual isolation contributes strongly under parapatry.

Discussion

Causes of host preference evolution

We examined the effects of three evolutionary processes on host preference evolution in *T. cristinae* walking-stick insects: (i) selection for reduced search costs and efficient host finding, (ii) selection against maladaptive host-switching and (iii) between-population gene flow. There is no direct evidence to support the 'search costs' hypothesis in *Timema* (i.e. search costs have not been measured), but nonetheless, allopatric populations clearly show differentiation in host preference. Habitat fidelity in the absence of fitness trade-offs between hosts has been detected in other systems (Futuyma *et al.*, 1984) and among the allopatric populations (Funk, 1998; Forister, 2004). These observations, coupled with the results of this study, show that active selection again maladaptive host-switching is not required for preference evolution.

Selection against maladaptive host-switching can also contribute to host preference evolution. Local adaptation, via divergent natural selection, results in performance trade-offs between alternative habitats. This process favours the evolution of divergent host preferences because individuals switching hosts are selected against. Fitness trade-offs between different habitats have been detected in a number of taxa (Schluter, 2000 for review), including host-associated insects (Blau & Feeny, 1983; Katakura *et al.*, 1989; Craig *et al.*, 1997; Carroll *et al.*, 1997; Via *et al.*, 2000), mollusks (Giesel, 1970; Rolan-Alvarez *et al.*, 1997), amphibians (Storfer & Sih, 1998; Storfer *et al.*, 1999) and fish (Schluter, 2000). In several of these cases, forms adapted to alternative habitats exhibit a preference for their native habitat, and thus are partially reproductively isolated (e.g. ladybird beetles, Katakura *et al.*, 1989; *Eurosta solidaginis*, Craig *et al.*, 1993; pea aphids, Via, 1999). These results suggest that fitness trade-offs commonly drive the evolution of divergent habitat preferences. However, explicit tests of this hypothesis are lacking – that is, there are few tests of host preferences in sympatric vs. allopatric populations (but see Forister, 2004).

Selection against host-switching is a process, and one predicted outcome of this process is increased divergence in sympatric vs. allopatric populations. However, this process need not always result in such 'character displacement' of host preference (e.g. Lemmon *et al.*, 2004). For example, selection against host-switching almost certainly occurs in *T. cristinae* (Nosil, 2004) and likely contributes to preference evolution because parapatric populations exhibit divergence in host preference in the face of gene flow and mean levels of divergence that are not drastically (nor always) lower than those observed for allopatric populations. Most likely, selection against switching between *Adenostoma* and *Ceanothus* contributes to preference evolution but greater relative divergence is not observed in parapatric populations because of gene flow in parapatry (which decreases parapatric divergence) and strong direct selection on preference in allopatry (which increases divergence in allopatry). Notably, character displacement of mate preferences has occurred in *T. cristinae* (Nosil *et al.*, 2003), perhaps because direct selection for mate preference is weak in allopatry and because insects can move whereas plants cannot (such that mating decisions occur more commonly than host picking decisions).

We found some evidence for an inverse association between the population divergence and gene flow. The results were likely not stronger because selection against host-switching in parapatry counters the homogenizing effects of gene flow. Our results indicate host preference evolution can indeed occur in the face of gene flow (see also Forister, 2004; Emelianov *et al.*, 2004), but that divergence might be somewhat constrained. The standard interpretation of an inverse association between gene flow and population divergence is that gene flow constrains divergence (Slatkin, 1987). However, causality can be reversed because adaptive trait divergence itself may reduce gene flow (i.e. 'ecological speciation' Schluter, 2000; Lu & Bernatchez, 1999; Hendry *et al.*, 2002; Hendry & Taylor, 2004; Hendry, 2004). In *T. cristinae*, both processes likely act. To some extent, gene flow must constrain divergence because divergence is greatest between allopatric populations, yet the adaptive divergence of allopatric populations cannot reduce contemporary gene flow between them (i.e. as they are geographically separated). In the parapatric scenario, host preference is likely to itself reduce gene flow (see section on RI below). Thus in *T. cristinae*, these two processes might be involved in a positive feedback loop whereby low gene flow allows adaptive divergence, which in turn further reduces gene flow by increasing RI (Hendry, 2004). An outstanding question is why this feedback has not resulted in greater divergence.

Multiple lines of evidence indicate that the population divergence in host preference detected in this study has a partial genetic basis. While our experiments do not unequivocally rule out maternal effects (Mousseau & Dingle, 1991), all of the available evidence indicates that genetic divergence has occurred such that there has been progress towards genetically differentiated host forms (rather than the evolution of plasticity).

Asymmetry in host preferences

The divergent host preferences detected in this study were atypical in that they were relative, not absolute. Thus, individuals from populations using either host plant often preferred to rest on *Ceanothus*, with walking-sticks collected from *Ceanothus* exhibited a much stronger preference for *Ceanothus* than those from *Adenostoma*. In parapatric populations where host choice is possible, this pattern might reflect the outcome of directional fecundity selection, which can counteract selection to prefer *Adenostoma*. Selection on colour-pattern is divergent and can indirectly cause the evolution of divergent host preference via the positive genetic association between colour-pattern and preference. In contrast, fecundity selection favours preference for *Ceanothus* independent of colour-pattern because females from both hosts exhibit higher fecundity on *Ceanothus* (Sandoval & Nosil, 2005). Thus, fecundity selection might constrain the evolution of strong preference for *Adenostoma*.

Additionally, the ancestral host of *T. cristinae* is not unequivocally known but it is possible that evolution away from an ancestral preference for *Ceanothus* is ongoing (Crespi & Sandoval, 2000). Thus, allopatric populations of *Ceanothus* may simply retain the ancestral preference and exhibit strong preference for their native host. Conversely, allopatric *Adenostoma* populations would not exhibit a strong preference for their native host as they are in the process of evolving away from the ancestral preference. This process could also explain the stronger association between gene flow and trait divergence detected in the *Ceanothus* vs. *Adenostoma* populations. Allopatric *Adenostoma* populations would not have evolved away from the ancestral preference and thus exhibit similar preferences to that of parapatric populations.

Evolution of means vs. genetic covariances

This study focused on divergence in population means. The evolution of trait means in response to natural selection depends on the genetic covariance between traits within populations (Lande, 1979; Arnold, 1992; Schluter, 1996). Thus, to better understand host preference evolution, genetic covariance between host preference and colour-pattern (a trait known to be under host-specific selection) has also been examined in a subset of the populations studied here (Nosil *et al.*, in press). At parapatric sites, divergent selection results in differentiation between adjacent populations on different hosts in both traits (Sandoval, 1994a,b; Nosil, 2004). Migration between hosts occurs and generates nonrandom associations between alleles at colour-pattern and host preference loci (linkage disequilibrium; Nei & Li, 1973; Kirkpatrick *et al.*, 2002), resulting in strong genetic covariance between colour-pattern and host preference. In allopatry, divergent selection and migration between hosts do not occur and genetic covariance is absent.

Collectively, these studies shed additional light on the mechanisms of population divergence because they show that genetic covariance need not result in greater population-level divergence. In *T. cristinae*, parapatric populations using different hosts show weaker population-level differentiation in both host preference and colour-pattern than do allopatric populations, despite stronger genetic covariance within the former. This result indicates that host preference can evolve in parapatric populations via indirect selection (i.e. because of direct selection on colour-pattern), but it also suggests that such indirect selection acting through imperfect genetic associations is a weak diversifying force compared with direct selection (Felsenstein, 1981; Kirkpatrick & Barton, 1997). Heterogeneous environments might promote divergence and sympatric speciation by favouring genetic covariance (Lande, 1979; Kawecki, 2004), but hamper speciation by exposing the populations to gene flow (Felsenstein, 1981; Slatkin, 1987).

Components of premating isolation

Coyne & Orr (2004, p. 57) state that 'the central problem of speciation is understanding the origin of those isolating barriers that actually or potentially prevent gene flow in sympatry'. They note that this involves two major tasks: determining which reproductive barriers were involved in the initial reduction in gene flow between populations and then understanding which evolutionary forces produced these barriers. Our results shed light onto both these issues. The observed divergence in host preference will cause partial (albeit relatively weak) premating isolation even though it is asymmetric because individuals from populations using different hosts should encounter one another less frequently than individuals from within the same host (Coyne & Orr, 2004). The host-associated forms of *T. cristinae* represent conspecific populations and thus three major forms of premating isolation (habitat isolation, immigrant inviability and sexual isolation) are involved in the initial divergence between populations. With respect to evolutionary forces, our studies show that selection facilitates divergence in all three forms of premating isolation examined, whereas gene flow constrains it (see also Nosil *et al.*, 2002, 2003; Nosil, 2004). Moreover, both host-specific selection for local adaptation and selection to avoid maladaptive hybridization/host-switching facilitate divergence, indicating that multiple forms of selection are involved in the evolution of reproductive barriers.

The host-associated forms of *T. cristinae* are unlikely to have achieved species status by any criterion, as indicated by only a 60% barrier to gene flow at the premating level (Fig. 4) and a general lack of neutral mtDNA differentiation between adjacent populations on different hosts because of ongoing gene flow (Nosil *et al.*, 2003). Thus, these host forms represent either an ongoing speciation event or population divergence that has reached equilibrium. Further studies of more divergent species within this genus may shed light onto the factors driving the transition from a host race or ecotype to a species. Clearly though, selection is central to divergence, indicating that the population-genetic processes acting within contemporary populations can also influence the formation of new species (Charlesworth *et al.*, 1982).

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Appendix 1 Host preference of *Timema cristinae* walking-sticks collected from populations using *Ceanothus spinosus* and *Adenostoma fasciculatum* as host-plants.

			Experiment #1		Experiment #2			Experiment #3		
Host	Pop.	% area *C*	# ind.	% picking *C*	# reps.	# ind.	% picking *C*	# fam.	# ind.	% picking *C*
C	p	100	149	93	–	–	–	10	77	57
C	hvc	34	29	79	–	–	–	6	10	25
C	vpc	100	174	91	–	–	–	1	2	50
C	pr	100	68	93	11	89	78	–	–	–
C	outc	67	21	90	–	–	–	–	–	–
C	gibr	100	34	94	–	–	–	–	–	–
C	pe	100	33	100	–	–	–	12	132	79
C	r12c	70	70	90	10	10	90	14	97	73
C	r6c	100	1	100	–	–	–	3	11	71
C	mboxc	5	52	87	–	–	–	12	50	67
C	vpwc	100	3	100	–	–	–	–	–	–
C	outwc	100	4	100	–	–	–	–	–	–
C	ogc	1	41	76	–	–	–	–	–	–
C	sc	100	1	100	–	–	–	4	16	83
C	wcc	100	3	100	–	–	–	6	43	82
C	r9c	90	–	–	11	11	73	–	–	–
C	ptc	54	–	–	7	24	51	–	–	–
C	vpac	94	–	–	–	–	–	1	6	67
C	mc	39	16	81	–	–	–	2	15	46
Total			699	–	39	144	–	71	459	
A	hva	34	98	78	7	75	66	17	78	40
A	ma	39	52	62	24	196	46	10	80	63
A	la	0	139	63	3	28	30	13	135	42
A	vpa	94	103	84	17	135	61	–	–	–
A	ha	8	111	81	–	–	–	–	–	–
A	outa	67	84	86	–	–	–	1	1	100
A	r12a	70	57	44	20	20	60	11	125	60
A	r23a	0	1	0	–	–	–	7	28	54
A	mboxa	5	68	60	7	58	66	7	30	74
A	oga	1	9	89	2	19	44	–	–	–
A	lrn	0	5	80	–	–	–	6	36	21
A	r21a	10	–	–	18	18	61	–	–	–
A	pta	54	–	–	6	27	43	–	–	–
A	loga	15	–	–	–	–	–	2	16	46
Total			727	–	104	566	–	74	529	–

Ind., individuals; reps., replicates; fam., families; pop., population. *C* = *Ceanothus*.