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ORIGINAL PAPER

Natural selection and divergence in mate preference during speciation

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Abstract Sexual isolation can evolve due to natural selection against hybrids (reinforcement). However, many different forms of hybrid dysfunction, and selective processes that do not involve hybrids, can contribute to the evolution of sexual isolation. Here we review how different selective processes affect the evolution of sexual isolation, describe approaches for distinguishing among them, and assess how they contribute to variation in sexual isolation among populations of *Timema cristinae* stick-insects. Pairs of allopatric populations of *T. cristinae* living on different host-plant species exhibit greater sexual isolation than those on the same host, indicating that some sexual isolation has evolved due to host adaptation. Sexual isolation is strongest in regions where populations on different hosts are in geographic contact, a pattern of reproductive character displacement that is indicative of reinforcement. Ecological costs to hybridization do occur but traits under ecological selection (predation) do not co-vary strongly with the probability of between-population mating such that selection on ecological traits is not predicted to produce a strong correlated evolutionary response in mate preference. Moreover, F1 hybrid egg inviability is lacking and the factors contributing to reproductive character displacement require further study. Finally, we show that sexual isolation involves, at least in part, olfactory communication. Our results illustrate how understanding of the evolution of sexual isolation can be enhanced by isolating the roles of diverse ecological and evolutionary processes.

Keywords Speciation · Hybrid fitness · Mate choice · Reproductive isolation · Pheromones

Introduction

Speciation involves the evolution of reproductive isolation between diverging populations. Understanding speciation thus requires determining which reproductive barriers initially reduced gene flow between populations and the evolutionary forces producing them (Mayr 1947, 1963; Coyne and Orr 2004). Barriers to gene exchange can occur before or after mating, and different forms of reproductive isolation are not necessarily independent. For example, selection against hybrids (postmating isolation) can drive the evolution of increased premating isolation (i.e. reinforcement; Dobzhansky 1937; Servedio and Noor 2003). The evolution of premating isolation caused by divergent mating signals and preferences (sexual isolation hereafter) appears to be an important component of speciation in many taxa (Coyne and Orr 2004). Many selective processes can affect the evolution of sexual isolation, but their relative contributions are poorly understood. In this paper, we describe selective processes which can promote the evolution of sexual isolation, present methods for distinguishing among them, and apply the methods to explain variation in sexual isolation among populations of walking-stick insects.

Reproductive isolation can evolve simply as a by-product of populations adapting to different

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ecological environments (Funk 1998; Schluter 2000; Jiggins et al. 2001, 2004; Podos 2001; Etges 2002; Richmond and Reeder 2002; Rundle and Nosil 2005; Funk et al. 2006; Vines and Schluter 2006). Divergent natural selection acts on ecologically important traits, resulting in population divergence in ecological traits. If these traits, or ones genetically correlated with them, incidentally affect mate choice, then sexual isolation evolves as a by-product of local adaptation. This is the classic 'by-product' model of allopatric speciation (Muller 1942), but it applies to any geographical scenario. Notably, by-product speciation invokes selection on ecological traits, and the resulting selection on mating traits can be either direct or indirect (Rundle and Nosil 2005). If the loci affecting selected and mating traits are the same (i.e. due to pleiotropy; Kirkpatrick and Ryan 1991), then direct selection occurs. (Schemske and Bradshaw 1999; Bradshaw and Schemske 2003) If the loci are physically different, then selection acts indirectly on mate preference alleles through their genetic association (i.e. linkage disequilibria) with alleles at other loci, which are under selection (Barton and Turelli 1991; Kirkpatrick 1996; Kirkpatrick and Barton 1997; Kirkpatrick and Servedio 1999; Servedio 2001, 2004). Such indirect selection acting through imperfect genetic associations is thought to be less effective at driving speciation than is direct selection (Kirkpatrick and Ravigne 2002).

Sexual isolation can also evolve due to direct selection on actual mate preferences, rather than selection on ecological traits per se (Servedio 2001). Selection on a preference is direct when the preference allele affects fitness independent of the genetic background in which it is found (Kirkpatrick and Ryan 1991; Kirkpatrick 1996; Kirkpatrick and Ravigné 2002). Simple examples are where preferences for detectable signals accrue high fitness or where preference results in greater parental investment from mating partners. If divergent environments differ in their signal transmission properties, direct and habitat-specific selection may be imposed on sensory systems and preferences (i.e. 'sensory drive'—Morton 1975; Ryan et al. 1990; Endler 1992; Boughman 2002; Slabbekoorn and Smith 2002; Seddon 2005; Patten et al. 2004; Fuller et al. 2005 for review). Preference evolution arising from such habitat-specific and direct selection on preferences can be thought of as a form of local adaptation. Thus both habitat-specific selection on preferences and the by-product model predict greater sexual isolation between ecologically divergent pairs of populations than between ecologically similar pairs of similar age.

Selection against hybrids can also result in the evolution of sexual isolation ('reinforcement' Dobzhansky 1937). During reinforcement, selection acts directly on genes causing low hybrid fitness, and mating preferences evolve via their genetic association with genes causing low hybrid fitness (i.e. via indirect selection). When populations or species hybridize, both selection against hybrids and the genetic association (disequilibria) between loci may be large, such that even indirect selection acting thru imperfect genetic associations is relatively strong (Kirkpatrick and Ravigné 2002). Initial work on reinforcement focused on intrinsic postmating isolation (hybrid inviability or sterility) resulting from between-locus genetic incompatibilities (Bateson 1909; Dobzhansky 1936, 1937; Servedio and Noor 2003; Gavrilets 2004). However, selection against hybrids can also occur without intrinsic postmating isolation. Hybrids may exhibit a poor ecological fit to the niches of parental species and such 'extrinsic hybrid inviability' can drive reinforcement (Fisher 1930; Hatfield and Schluter 1999; Kirkpatrick 2001; Orr and Turelli 2001; Rundle and Whitlock 2001; Rundle 2002; Servedio 2004). Likewise, sexual selection against hybrids can contribute to reinforcement (Phelan and Baker 1987; Noor 1997; Vamosi and Schluter 1999; Naisbit et al. 2001). Finally, costs to hybridization that do not involve hybrids can affect preference evolution. For example, hybridization can reduce the survival or fertility of females themselves, favoring the evolution of sexual isolation (i.e. these are the postmating, prezygotic incompatibilities of Howard and Gregory 1993; Servedio 2001).

These considerations indicate that numerous selective processes can contribute to the evolution of sexual isolation, and the relative importance of different processes is a major outstanding question in speciation research. We do not exclude a role for processes that were not mentioned, such as genetic drift, sexual selection, or sexual conflict (Ryan and Rand 1993; Panhuis et al. 2001; Shaw and Parsons 2002; Gavrilets 2004). Rather, we focus on local adaptation and selection against hybridization because they (1) appear common in nature (Coyne and Orr 2004), (2) are the topic of recent yet generally untested theory, and (3) apply to our study system, *Timema cristinae* walking sticks (drift and environment-independent sexual selection have been examined, but evidence suggests they do not contribute strongly; Table 1). Speciation is often a continuous process, with different 'stages' corresponding to the magnitude of reproductive isolation that has evolved. Ecology and geography dictate which processes act, and genetics influences the response to selection (Fig. 1).

Table 1 Summary of the evolutionary processes involved in the evolution of sexual isolation among populations of *T. cristinae* walking-stick insects

Evolutionary process	Occurs?	Contributes?	Explanation
Local adaptation	Yes	Yes	Allopatric populations using different hosts show greater sexual isolation than those using the same host
Direct selection/sensory drive	Possible, evidence lacking	Possible	Trait causing postmating incompatibilities does not covary strongly with mate preference, leaving a potential role for direct selection
Genetic drift	Yes	No (at least not in isolation)	Levels of sexual isolation are uncorrelated with divergence in neutral markers
Environment-independent sexual selection	Unknown	No (at least not in isolation)	Different populations which use the same host exhibit little or no sexual isolation
Postmating, prezygotic incompatibilities			
Female survival	Yes	Possible	Selection occurs, but male trait reducing female survival does not covary strongly with preference
Female fecundity	Maybe	Maybe	Not certain if selection occurs
Selection against hybrids			
Intrinsic F1 egg inviability	No	No	Does not occur, so cannot contribute
F1 sterility, F2 and backcross breakdown	Unknown	Unknown	Not examined
Natural selection	Yes	Possible	Selection occurs, but trait reducing hybrid survival does not covary strongly with preference
Sexual selection	Likely	Unknown	Never been measured, but likely given divergent mate preferences/mating traits of parental forms

Shown is whether an evolutionary process occurs at all, and then if it contributes to the evolution of sexual isolation

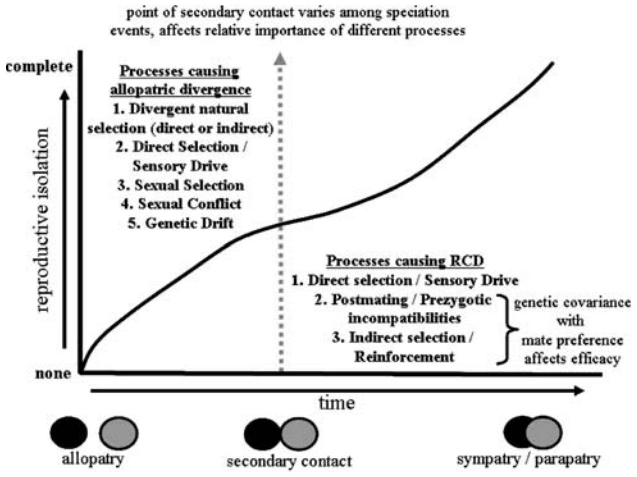

Fig. 1 A scenario for how speciation may progress from its earliest to latest stages (magnitude of reproductive isolation is depicted by the wavy, black line). The scenario shown here applies to the evolution of sexual isolation, and lists the potential processes involved. Populations are initially allopatric, but secondary contact can occur at any point and results in additional processes which can drive divergence. In the particular case shown here, secondary contact occurs roughly midway through speciation—depicted by the dashed vertical line. When indirect selection occurs, the genetic covariance between traits affecting mate preference and traits under selection is important for evolution. See the Introduction for details and Table 1 for processes that have been most important for the evolution of sexual isolation among populations of *T. cristinae* walking-sticks. RCD refers to reproductive character displacement. The figure is modified from Rundle and Nosil (2005)

Distinguishing among selective processes involved in speciation

A first step towards distinguishing among processes is to compare sexual isolation among populations with varying degrees of ecological divergence (a proxy for local adaptation) and geographic potential for reinforcement (Funk et al. 2002). Further steps involve elucidating the costs to hybridization potentially driving reinforcement, quantifying the association between selected traits and mating preferences (which affects the strength of indirect selection transmitted to mate preferences), and determining the traits upon which sexual isolation is based. We describe these steps in more detail below, and apply them to populations of *Timema* walking-sticks. Some of our inferences are based upon indirect evidence but we nonetheless dissect the role of multiple selective processes in the evolution of sexual isolation in the context of an explicit body of theory (Table 1 for summary).

Timema walking-stick study system

Timema walking-sticks are wingless, phytophagous insects inhabiting the chaparral of Southwestern North America (Crespi and Sandoval 2000). Individuals feed and mate exclusively on the hosts upon which they rest. We focus on *T. cristinae*, a species feeding upon two different host-plant species (*Ceanothus spinosus* and *Adenostoma fasciculatum*). We define a 'population' of walking-sticks as all the individuals collected within a homogenous patch of a single host species (as in Nosil et al. 2002, 2003). Thus 'parapatric' insect populations are in contact with an 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 each population had only one adjacent population on the alternate host. For simplicity, we use the term 'hybridization' to refer to interbreeding between populations on different hosts, but do not imply that the host forms have achieved species status.

In this study we present much new data, but also re-analyze and re-evaluate some previously published data in order to synthesize the collective findings. The data novel to this paper include: (1) analyses of pheromones and behavioral responses to them, (2) genetic crosses examining hybrid egg inviability, (3) field collections examining the fitness of different mating-pair types in nature, and (4) rearing experiment testing for a heritable basis to population divergence in color-pattern. By contrast, all mate preference data stem from previously published mate-choice experiments, and the examination of covariance between color-pattern and mate preference in the current study involves a novel analysis of these data (Nosil et al. 2002, 2003). Collectively, the different types of data provide insight into the divergence of mating preferences during the early stages of speciation. We first review previous evidence for the role of host-adaptation and reinforcement in the evolution of sexual isolation. We then present new results on the costs to hybridization potentially driving reinforcement and the traits affecting sexual isolation.

Host-adaptation and the evolution of sexual isolation

If local adaptation drives divergence, then sexual isolation is predicted to be greater between ecologically divergent pairs of populations than between ecologically similar pairs of similar age. Several studies have provided evidence for such a pattern (Funk 1998; Rundle et al. 2000; Cruz et al. 2004; McKinnon et al. 2004; Funk et al. 2006; Vines et al. 2006). In *T. cristinae*, a role for host-adaptation is implicated by the observation that sexual isolation is stronger between geographically separated pairs of populations using different host-plant species than between geographically separated pairs of populations using the same host (Nosil et al. 2002). Conversely, sexual isolation is uncorrelated with divergence in mitochondrial and nuclear DNA (COI and ITS-2, respectively) between these populations. Thus divergence in host plant use, rather than neutral differentiation via genetic drift, is the predictor of sexual isolation. The weak sexual isolation between populations using the same host also indicates that environment-independent forms of sexual selection do not strongly affect sexual isolation. Because selection against hybridization cannot occur when populations are geographically separated, a role for local adaptation is inferred. We note that sexual isolation likely has a strong heritable genetic basis because it is unaffected by rearing environment (i.e. *Ceanothus* versus *Adenostoma*, Nosil et al. 2003).

Reinforcement and reproductive character displacement

Reinforcement predicts reproductive character displacement: increased sexual isolation in geographic regions where hybridization occurs (sympatry/parapatry) relative to regions where it does not (allopatry; but see Lemmon et al. 2004). Several cases of reproductive character displacement have been documented (Wasserman and Koepfer 1977; Zouros and d'Entremont. 1980; Noor 1995; Saetre et al. 1997; Rundle and Schluter 1998; Higgie et al. 2000; Hobel and Gerhardt 2003; Albert and Schluter 2004; Hoskin et al. 2005), yet few studies have distinguished among alternative hypotheses to reinforcement, or the role of different types of low hybrid fitness (Butlin 1995; Servedio and Noor 2003 for review). This is not a trivial task because different processes are not mutually exclusive and often generate overlapping predictions (Day 2000; Servedio and Noor 2003). For example, novel signals might be favored in a new environment to reduce overlap with signals in the ancestral environment (Wasserman and Koepfer 1977). In this scenario, 'competition' among signals occurs only in parapatry such that direct selection on the preference (rather than reinforcing selection) would generate reproductive character displacement (Servedio 2001; Boughman 2002; Slabbekoorn and Smith 2002).

In addition to the effects of host adaptation, there is evidence for reinforcement in *T. cristinae*. Female mating discrimination against males from the alternative host is stronger when populations from different hosts are in geographic contact than when they are fully allopatric (Nosil et al. 2003). This pattern of reproductive character displacement could have evolved in response to maladaptive hybridization between the host-forms because (1) gene flow and interbreeding occurs, (2) alternative explanations such as ecological character displacement, differential fusion of populations, and population ancestry were examined, but are unsupported, and (3) indirect evidence suggests that hybrids will suffer low fitness due to high rates of visual predation. However, costs to hybridization have not been systematically evaluated in previous studies. Moreover, the association between selected traits and mating preferences affects the potential for reinforcement, but has also not been examined. In this paper, we present preliminary data on these two factors, and discuss their potential role in driving divergence in parapatric populations.

Costs to hybridization potentially contributing to reinforcement

A clear step beyond simply documenting reproductive character displacement is to ascertain which forms of selection drove preference evolution. Multiple forms of postmating isolation are measured, as well as costs to hybridization for individuals themselves. If individual or hybrid fitness is not reduced by a particular mechanism, a critical role for this process is unlikely. Here we estimate two costs to hybridization, intrinsic hybrid egg inviability and reduced survival of hybridizing females due to predation. We also evaluate the potential for extrinsic reductions in hybrid fitness.

(1) Intrinsic hybrid egg inviability (hatching success)

We tested for intrinsic F1 hybrid egg inviability using within-population and between-population crosses (Table 2 for population pairings; $n = 607$ crosses). Nymph *T. cristinae* were field-collected from 28 populations in the Santa Ynez Mountains, California in spring 2003 and 2004 using sweep nets. They were reared in glass jars (20°C) with 10–15 individuals per jar at the University of California at Santa Barbara. All individuals used in the crosses were sexually immature instars that were reared to sexual maturity on *Ceanothus* cuttings. Individuals from different populations, and males and females, were kept separate during rearing. Within two days of attaining sexual maturity, a single virgin male and a single virgin female were housed together in a Petri dish and observed until they mated. Then they were fed *Ceanothus* cuttings every second day until the female died (females lay eggs singly).

Hatching success was quantified from egg shell ($n = 30,958$ eggs) characteristics. Hatching nymphs

Table 2 Hatching-success was not reduced for between-population versus within-population crosses

Population pair	Within-population	Between-population	*n*	*F*-ratio	*p*-Value
Allopatric same hosts					
P × PR	32 (20)	34 (29)	56	0.38	0.54
PE × WCC	50 (33)	65 (25)	49	2.47	0.12
LOG × BT	41 (55)	42 (32)	11	0.11	0.74
Parapatric different hosts					
HVA × HVC	48 (34)	54 (29)	72	0.09	0.77
MA × MC	23 (26)	49 (30)	30	10.26	0.004
HA × HC	41 (28)	57 (18)	14	0.98	0.34
OUTA × OUTC	44 (32)	53 (30)	51	1.90	0.17
R12A × R12C	40 (40)	44 (35)	55	0.11	0.74
MBOXC × MBOXA	45 (37)	44 (24)	38	0.01	0.92
OGC × OGA	54 (42)	46 (28)	28	0.03	0.88
VPA × VPAC	54 (33)	25 (34)	44	7.36	0.01
Allopatric different hosts					
LA × VPC	65 (35)	49 (34)	50	1.11	0.30
R6C × R23A	40 (41)	47 (30)	66	0.49	0.49
SC × LRN	53 (39)	65 (26)	43	0.40	0.53

The table shows mean % (s.d.) hatching success for between-population and within-population crosses for 14 different pairs of *T. cristinae* walking-stick populations. Also shown are the number of crosses for each population pair and the test statistic for the male population by female population interaction term (this *F*-ratio stems from a separate ANOVA analysis for each population pair such that the degrees of freedom are $F_{1,n}$ where *n* refers to the total sample size) left the egg shell fully intact except for a small opening at one end. Thus the proportion of hatched eggs within a brood can be calculated by counting the number of eggs with or without a small opening at one end. Hatching success measured this way was highly correlated with hatching success measured by monitoring broods daily for newly hatched nymphs ($r = 0.99$, $p < 0.001$, $n = 12$ broods). The repeatability of hatching success estimates based on egg case characteristics was also estimated by recounting some broods in the same year and in a subsequent year. Statistical analyses were conducted on egg number from the year of initial count, in case some eggs were lost or damaged. This does not affect our results because hatching success was highly repeatable both within years ($r = 0.97$, $p < 0.001$, $n = 28$) and between years ($r = 0.95$, $p < 0.001$, $n = 107$).

In *T. cristinae*, most viable eggs overwinter in diapause and hatch the year after mating occurred. Thus our analyses focus on hatching success following one over-wintering. However, a small portion of eggs diapause over one additional winter. There is no reason to suspect that variation in the duration of diapause would differ for within-population versus between-population crosses, and thus hatching success following one over-wintering likely provides an unbiased measure of egg viability. However, to ensure that our data are not affected by variable diapause, we estimated hatching success for 104 broods after a 2-year (2003–2005), instead of 1-year (2003–2004), diapause. These data are also presented.

Two types of ANOVA were used to analyze hatching success. The first analysis pools data among different population pairs and examines whether hatching success varied among the four main types of crosses (within-population, between populations using the same host, between parapatric populations using different hosts, and between allopatric populations using different hosts). Year (2003 or 2004) was also included as a factor. Mean hatching success was similar for all types of crosses (within population crosses = 49%, s.d. = 31; between populations using the same host = 40%, s.d. = 28; between parapatric populations using different hosts = 45%, s.d. = 35; between allopatric population using different hosts = 51%, s.d. = 39). Thus there was no evidence for reduced hatching success of F1 individuals from between-population crosses (main effects of cross-type $F_{3,607} = 1.22$, $p = 0.30$, main effects of year $F_{1,607} = 0.07$, $p = 0.80$, year × cross-type interaction $F_{3,607} = 1.10$, $p = 0.35$; main effects of cross type in an analysis excluding the interaction term $F_{3,607} = 0.54$, $p = 0.65$). Congruent results were obtained when hatching success was calculated after two, rather than one, over-wintering (within population crosses = 70%, s.d. = 21, $n = 56$; between parapatric populations using different hosts = 72%, s.d. = 23, $n = 48$; main effects of cross-type $F_{1,103} = 0.36$, $p = 0.55$).

The analyses above pool the results among population pairs and are general and useful because they yield very large sample sizes for analysis and do not assume independence of population pairs. However, analyses on pooled data might obscure trends in individual population pairs and do not treat population pairs as the unit of replication. The second set of analyses alleviate these problems in two ways. First, we conducted a separate ANOVA for each of the 14 population pairs. The model for each individual population pair included male population, female population, and the interaction between male and female population. We are interested primarily in the interaction term which tests whether hatching success is dependent on population of origin for the two sexes (e.g. the same population or not). Examination of individual pairs of populations confirmed the absence of F1 hybrid egg inviability (Table 2). The interaction between male population and female population was significant ($p = 0.01$) for only one of the 14 population pairs, and this difference is insignificant following Bonferroni correction. Second, we examined trends among population pairs (i.e. using population pairs as the unit of replication). There were no directional trends evident such that only four of 14 pairs showed reduced hatching success in between-population versus within-population crosses ($p = 0.82$, Binomial test). Thus strong, F1 hybrid egg inviability was not detected in our study, and is therefore unlikely to strongly contribute to reinforcement in this system.

Intrinsic inviability might be more pronounced in F2 or backcrossed individuals, which were not examined. Furthermore, hybrid sterility generally evolves prior to inviability (Coyne and Orr 2004) and was also not examined. However, even if intrinsic inviability occurs in advanced hybrids, or if hybrid sterility occurs, it is likely incomplete because there is evidence for ongoing gene flow between the host forms (Nosil et al. 2003). Thus, at the very least, our data indicate that F1 hybrids do not show intrinsic inviability as represented by hatching success. This is the level of hybrid breakdown examined in most comparative studies of the evolution of reproductive barriers (reviewed in Coyne and Orr 2004; see also Mendelson 2003). Whether there has been sufficient time for hybrid egg inviability to evolve cannot be answered without studying more divergent taxa in the genus *Timema* (Crespi and Sandoval 2000; Sandoval and Nosil 2005). We note that there has been sufficient time for other forms of reproductive isolation to evolve in this species and that allopatric pairs of populations of *T. cristinae* exhibit substantial divergence in mitochondrial and nuclear DNA, indicative of reasonably ancient divergence times (Nosil et al. 2002).

(2) Ecological costs to hybridization I—survival of females

Low survival of females that mate with males from the alternative host could also select for avoidance of between-host mating. The survival of females could be affected by the color-pattern of the males riding on their back. *T. cristinae* exhibits two main color-pattern morphs. Both morphs occur on both host species, but relative frequencies have diverged between hosts such that the unstriped morph is more common on *Ceanothus* (mean frequency = 81%), whereas the striped morph is more common on *Adenostoma* (mean frequency = 72%). Thus populations on different hosts show consistent, but not fixed, differences in morph frequency. Population divergence has occurred via differential visual predation: the unstriped color-pattern confers high survival on *Ceanothus* but low survival on *Adenostoma*, and vice versa for the striped pattern (Sandoval 1994a, b; Nosil 2004; Nosil et al. 2005).

Population divergence in color-pattern morph frequencies can result in a cost to between-population mating, particularly for females. In *T. cristinae*, males ride on the back of the female during and following mating. This period where a male rides on a female's back lasts at least several hours in the field (Nosil unpublished) and several days under laboratory conditions (Nosil et al. 2003). Most individuals within a population are the locally cryptic color-pattern morph. Conversely, many immigrant males from the adjacent population on the alternative host are the locally less-cryptic morph. Thus females that mate with and carry less-cryptic immigrant males on their back are likely to suffer higher rates of visual predation than females who mate with cryptic males from their own population. This reduction in survival of hybridizing females could favor the evolution of increased mating discrimination against males from the alternative host.

The scenario outlined above requires reduced survival of females mating with less-cryptic males. Studies of individuals (rather than mating pairs) suggest that this will occur (Sandoval 1994a, b; Nosil 2004), but the fitness of mating pairs themselves has not been examined. We tested for selection against less-cryptic mating pair types in natural populations. If selection against a pair type occurs, its relative frequency should decline through time (i.e. between sequential sample periods; Endler 1986). In our analyses, the sequential sample periods are successive months. We have used these procedures previously to test for selection against the rare, intermediate morph (Nosil et al. 2003). In 2004, we collected mating pairs at one site that contained a population on each host (Refugio 12). We sampled adult insects in April and May 2004 (which represent periods before and after selection, respectively), recording the color-morph combination of each mating pair (total $n = 174$ mating pairs). For each host separately, we assessed whether the frequency of pair-types differed between months (i.e. before and after selection) using χ^2-tests. We also tested whether the change in pair types was dependent on host species, using a loglinear analysis. This analysis used a partial χ^2 value to assess the significance of the three-way interaction between pair-type, month and host (Norusis 1993).

We detected evidence for natural selection against less-cryptic mating pairs in natural populations (Fig. 2). Thus the relative frequency of the four different male/female pair types changed between months (i.e. before versus after selection) and, as expected, the nature of these changes was significantly dependent on host-plant species (pair-type × month × host interaction, $\chi^2 = 8.94$, d.f. = 3, $p < 0.05$, loglinear). On both hosts, mating pairs where both sexes were the cryptic morph increased after selection (thus striped/striped increased on *Adenostoma* and unstriped/unstriped increased on *Ceanothus*). Conversely, pairs where both sexes were the less-cryptic morph decreased after selection. However, differences before and after selection were statistically significant only for *Ceanothus* ($\chi^2 = 8.78$, d.f. = 3, $p < 0.05$, $n = 97$; for *Adenostoma*, $\chi^2 = 5.50$, d.f. = 3, $p = 0.14$, $n = 77$). Selection against less-cryptic mating pairs likely occurs and represents a cost to hybridization potentially contributing to reproductive character displacement.

Although our method was the only way to assess selection using undisturbed, natural populations, it potentially confounds differential survival with differential dispersal (Endler 1986). We note, however, that the results of manipulative experiments have demonstrated selection against less-cryptic individuals (Sandoval 1994a; Nosil 2004), increasing the likelihood that our current results represent selection against less-cryptic mating pairs. The reduced survival of hybridizing females is more likely indirect than direct selection, because the cost to females depends on the traits (color-pattern in this case) males and females carry (Servedio 2001, p. 1913 for explicit discussion of this issue). Thus, genetic covariance between color-pattern and mate preference is required for mate preference

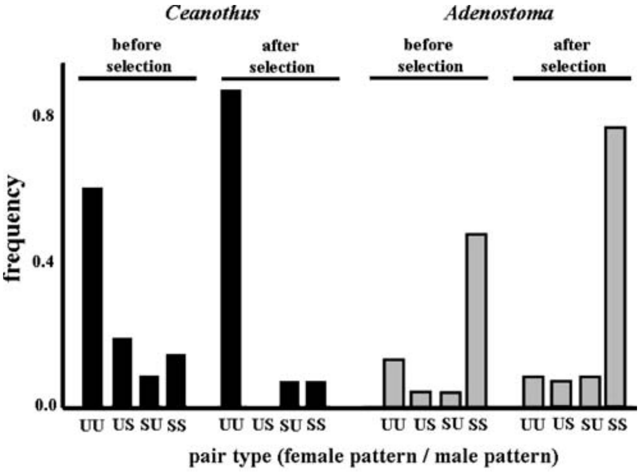

Fig. 2 Analyses of natural selection using *T. cristinae* mating pairs in the wild show selection against the locally less-cryptic mating pairs; on both hosts, mating pairs with a cryptic male and female morph increased after selection (i.e. between successive sample months). Thus ‘‘striped/striped’’ pairs increased on *Adenostoma* and ‘‘unstriped/unstriped’’ pairs increased on *Ceanothus*. Conversely, pairs with a less cryptic male and female morph decreased. UU = both sexes unstriped, US = unstriped male on striped female, SU = striped male on unstriped female, SS = both sexes striped

evolution due to direct selection on color-pattern. This covariance is assessed in a subsequent section.

(3) Ecological costs to hybridization II—survival of hybrids

Even if hybridizing females survive and produce offspring, ecologically based natural selection against hybrids could drive reinforcement. We were unable to assess directly the fitness of hybrids in the wild. Instead, we inferred the possibility of ecological selection against hybrids using estimates of selection on color-pattern and inferences about the color-pattern of hybrids. As noted, populations on different hosts exhibit divergent color-pattern morph frequencies such that each morph is more common on the host upon which it has higher survival. If population divergence in color-pattern has a strong genetic basis, then it is likely that hybrids will suffer reduced fitness due to exhibiting maladaptive color-patterns. Under additive inheritance of color-pattern, hybrids will exhibit low survival because intermediate morphs (which bear a faint stripe and occur at low frequency in the wild) have lower survival on both hosts than does the locally cryptic morph (Nosil et al. 2003; Nosil 2004). Under dominance, hybrids will still exhibit lower survival on average, because hybrid broods have a higher tendency to be ‘mixed’ such that they exhibit both color-pattern morphs (rather than only the locally cryptic one; Sandoval 1993; Nosil et al. 2003). It is unclear which scenario applies because dominance in within-population genetic crosses is incomplete and between-population crosses are lacking. Because of this ambiguity, we cannot yet provide a quantitative estimate of hybrid fitness. Rather, we demonstrate that population divergence in color-pattern has a strong heritable basis and note that population divergence in color-pattern is predicted to result in low hybrid fitness under both modes of inheritance. To the extent that hybrids are intermediate, or to which hybrid broods contain both color-pattern morphs, the fitness of hybrid offspring will be reduced relative to offspring derived from within-population mating.

We tested for a heritable basis to population divergence in color-pattern using a reciprocal rearing experiment. For nine populations, we raised field-collected first instar nymphs ($n = 1859$; 4–6 weeks of rearing) to sexually mature adults on their native or alternative host (Fig. 3 for population-specific sample sizes). We used logistic regression analyses to test whether color-pattern (proportion of striped individuals) in these populations was affected by genotype, rearing environment (host plant 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

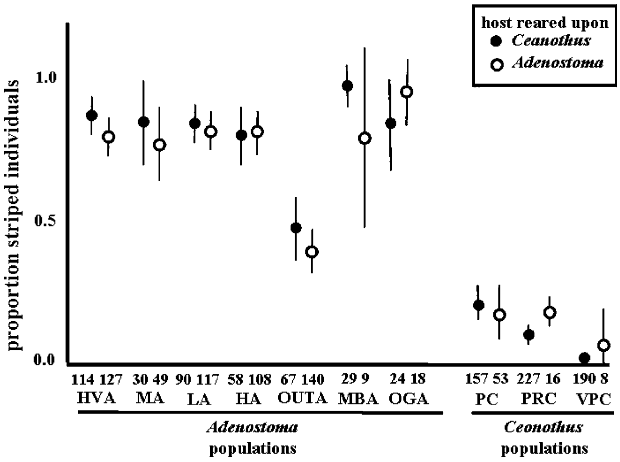

Fig. 3 Reciprocal rearing experiment demonstrates a heritable basis to population divergence in color-pattern. Shown is the proportion of striped adults (±95% C.I) observed when field-caught first instar *T. cristinae* were reared to adults during 4–6 weeks on *Ceanothus* (C) or *Adenostoma* (A). Populations adapted to *Adenostoma* are depicted on the left, populations adapted to *Ceanothus* are on the right. Genotypic effects (population and host of origin) were large and highly statistically significant (see Results for statistics). Values below the *x*-axis refer to number of individuals

genotype term. We report the results from a full model that included both factors and the interaction between them as well as the results from a reduced regression model derived using backward elimination (initial model included both factors and the interaction term but then removed all terms for which the significance of –2 log LR was >0.10).

The reciprocal-rearing experiment revealed that population divergence in color-pattern has a strong heritable basis (Fig. 3). A strong effect of genotype occurred when host of origin was used as the genotype term (full model, host of origin –2LR = 124.71, $p < 0.001$; host reared upon –2LR = 17.05, $p < 0.001$; interaction –2LR = 12.82, $p < 0.001$) and when population of origin was used as the genotype term (full model, population of origin –2LR = 10.15, $p < 0.01$; host reared upon –2LR = 49.12, $p < 0.001$; interaction term –2LR = 2.32, $p = 0.13$; reduced model, population of origin –2LR = 62.91, $p < 0.001$; host reared upon –2LR = 56.27, $p < 0.001$; interaction term removed). Although rearing effects were also detected, genotypic effects were consistent and large in magnitude. Heritable population divergence in color-pattern suggests that hybrid offspring will be less-cryptic such that selection against them could contribute to reinforcement. Evidence for reduced survival of hybridizing females themselves was presented above. Thus ecological costs to hybridization likely occur. The potential for these costs to drive the evolution of divergent mate preferences depends on the association between ecologically selected traits and mating preferences.

The association between selected traits and mate preferences

Direct selection on a trait generates indirect selection on correlated traits, and potentially a correlated evolutionary response. Another step in understanding preference evolution is to examine the magnitude of the association between traits under direct selection (e.g. ecological selection) and mate preference. Evolution of mate preferences by indirect selection depends on the magnitude of this genetic association. If the association is weak, then direct selection on mate preferences themselves may be a more likely cause of preference evolution (Kirkpatrick and Barton 1997).

In *T. cristinae*, for direct selection on color-pattern to cause the evolution of between-population mate preference, a genetic association must exist between color-pattern and the probability of between-population mating. We have previously shown that the probability of between-population mating is independent from whether both sexes are the same or different morphs (Nosil et al. 2002). These analyses test for assortative mating by color-pattern, but do not explicitly test for a genetic association between color-pattern and mate preference per se. A genetic association between these traits would be indicated by a difference between color-pattern morphs within each sex in the probability of between-population mating (i.e. a difference in whether each morph accepts or rejects individuals from the alternate host). We would expect that the more-cryptic morph is less-likely to hybridize in between-population mating trials. These predictions have not been tested previously, and are examined here using the mating trials from Nosil et al. (2002, 2003).

The previous studies examined mate choice using no-choice mating trials. A single male and a single female were housed in a Petri dish and whether copulation occurred within a 1-hour period was recorded. We employed logistic regression to test whether the probability of copulation in these trials was dependent on color-pattern morph. Our prediction concerns the probability that different color-pattern morphs within populations will hybridize with individuals from other populations. Thus, our analyses are restricted to the between-population mating trials and we conducted separate analyses for males and for females within each of the 12 populations from Nosil et al. (2002, 2003). We detected little or no evidence for a phenotypic association between color-pattern and mate preference. Thus color-pattern morphs did not tend to differ in the probability of between-population copulation when either males or females were considered (Table 3). Significant differences between color-pattern morphs were detected in only two of 24 cases, one was in the direction opposite to that predicted (i.e. cryptic morph more likely to hybridize), and neither was significant following Bonferroni correction (i.e. $p = 0.024$, 0.032 before correction for 12 comparisons within each sex).

Because the association between color-pattern and mate preference is weak, selection on color-pattern (in hybrids or hybridizing parental forms) is not predicted to cause a strong correlated evolutionary response in mate preference. Indirect selection on mate preference, generated by direct selection on color, will be weak. Thus how strongly the observed ecological costs to hybridization contribute to reproductive character displacement remains unclear. The observed lack of covariation between color-pattern and between-population mate preference is surprising because migration between populations should generate non-random association between alleles at different loci (i.e. linkage

Table 3 The proportion of between-population mating trials resulting in copulation for different color-pattern morphs of *T. cristinae*. The probability of copulation did not tend to differ between color-pattern morphs. PC, VPC, PRC and LA are allopatric populations, whereas the others are parapatric. Also shown is the likelihood ratio from likelihood ratio tests (all d.f. = 1)

Population	Unstriped mean (s.d.)	Striped mean (s.d.)	Unstriped *n*	Striped *n*	LR	*p*-Value
Males						
PC	0.32 (0.47)	0.29 (0.46)	156	58	0.15	0.70
HVC	0.26 (0.44)	0.36 (0.48)	54	59	1.24	0.27
VPC	0.30 (0.46)	0.13 (0.35)	223	15	2.19	0.14
PRC	0.29 (0.45)	0.00 (0.00)	168	1	0.67	0.41
MBOXC	0.27 (0.45)	0.29 (0.46)	26	174	0.06	0.80
OGC	0.18 (0.39)	0.17 (0.38)	78	86	0.01	0.93
HVA	0.49 (0.50)	0.35 (0.48)	98	145	4.61	0.03
VPA	0.33 (0.47)	0.29 (0.46)	148	66	0.40	0.53
MA	0.25 (0.44)	0.26 (0.44)	48	121	0.01	0.93
LA	0.23 (0.43)	0.26 (0.44)	52	162	0.17	0.68
HA	0.35 (0.48)	0.36 (0.48)	48	192	0.01	0.95
OUTA	0.29 (0.45)	0.34 (0.48)	153	73	0.69	0.41
Females						
PC	0.30 (0.46)	0.35 (0.48)	263	54	0.63	0.43
HVC	0.19 (0.40)	0.22 (0.42)	27	46	0.11	0.74
VPC	0.33 (0.47)	0.31 (0.47)	282	29	0.06	0.80
PRC	0.36 (0.48)	0.50 (0.71)	225	2	0.16	0.69
MBOXC	0.15 (0.36)	0.19 (0.40)	98	84	0.45	0.50
OGC	0.30 (0.46)	0.20 (0.40)	69	97	2.57	0.11
HVA	0.25 (0.44)	0.31 (0.46)	92	173	0.95	0.33
VPA	0.33 (0.47)	0.30 (0.46)	147	57	0.15	0.70
MA	0.00 (0.00)	0.23 (0.42)	11	48	5.09	0.02
LA	0.46 (0.50)	0.38 (0.49)	69	128	1.46	0.23
HA	0.34 (0.48)	0.32 (0.47)	38	135	0.08	0.78
OUTA	0.36 (0.48)	0.31 (0.47)	145	89	0.48	0.49

disequilibrium), even when they are physically unlinked (Nei and Li 1973; Kirkpatrick et al. 2002). The current study examined the phenotypic covariance between color-pattern and mate preference, whereas the most relevant parameter for evolutionary response is genetic covariance. The degree of correspondence between phenotypic and genetic covariance remains a topic of debate (Cheverud 1988; Willis et al. 1991; Arnold 1992; Schluter 1996). If the phenotypic covariance is not representative of the underlying genetic covariance, an undetected association at the genetic level could occur and have driven reinforcement. Moreover, recombination erodes genetic covariance caused by linkage disequilibrium, potentially accounting for the lack of covariance observed. Although further work is required, examining the association between color-pattern and mate preference was useful because it prevented us from concluding prematurely that ecological costs to hybridization (which appear strong) clearly drove reproductive character displacement.

Given that the available evidence suggests that indirect selection on mate preference will be weak, a role for direct selection on mate preference in the reproductive character displacement of mate preferences becomes highly plausible (Kirkpatrick and Barton 1997). Further studies in *Timema* could focus on direct selection on preference and signals, particularly now that candidate signals (pheromones) are known.

Traits affecting sexual isolation

A different approach to understanding sexual isolation, which is complementary to studies of geographic variation, is to identify the traits affecting mate choice (Nagel and Schluter 1998; Jiggins et al. 2001). Determining such traits allows subsequent studies of how selection acts on them, and thus how selection affects the evolution of sexual isolation (Hobel and Gerhardt 2003; Boughman et al. 2005). Surprisingly, there are relatively few systems where both geographic variation and the key traits affecting sexual isolation have been studied (reviewed in Coyne and Orr 2004; but see Higgie et al. 2000; Cruz et al. 2004; McKinnon et al. 2004; Boughman et al. 2005 for some exceptions).

Previous studies did not identify the traits affecting sexual isolation in *T. cristinae*. For example, between-population mating probability is independent of differences between the sexes in color-pattern, body size, and body shape (Nosil et al. 2002; Nosil and Crespi 2004). Here we present evidence from pheromone analyses and behavioral experiments that sexual isolation between populations stems, at least in part, from divergence in pheromones and responses to them. Successful copulation in *T. cristinae* involves at least two distinct stages. First, the male must approach the female and attempt to mount her. Second, the female must allow the male to mount and copulate with her, as males cannot force copulation. Here we test whether pheromonal communication affects the initial attraction of males to females, and thus has a potential role in sexual isolation.

We focus on two population pairs comprised of three populations. Thus a population adapted to *Adenostoma* (HVA) is compared to a population feeding on the same host plant (OUTA) and to a population feeding on *Ceanothus* (PR). The pair of populations using different hosts exhibits stronger sexual isolation than the pair using the same host (Nosil et al. 2002, 2003 for details). For example, using an index of sexual isolation (I_{PSI}) that ranges from –1 to +1 (with zero and one indicating random mating and complete assortative mating, respectively; Rolan-Alavarez and Cabarello 2000), $I_{\mathrm{PSI}} = 0.20$ for HVA × PR versus $I_{\mathrm{PSI}} = 0.11$ for HVA × OUTA. Averaged across numerous pairs of populations, mean $I_{\mathrm{PSI}} = 0.24$ and 0.08 for pairs on different versus the same host, respectively.

Methods for analyzing pheromones and behavioral responses

Our examination of pheromonal communication contained two main components; analytical procedures aimed at pheromone identification and behavioral experiments. To obtain pheromone, 16–24 male or female HVA or PR were placed in separate Pyrex glass chambers (5 × 10 cm) maintained at a photoperiod of 14L:10D and a temperature of 23–25°C. A water aspirator was used to draw humidified, charcoal-filtered air at a rate of 80 ml/min through the chamber and through a glass column (6 × 30 mm) filled with Porapak Q (50–80 mesh, Waters Associated Inc. Milford, MA, USA). After 40 h, absorbed volatiles were desorbed with 2 ml of redistilled pentane.

Pheromone extract was analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) and GC–mass spectrometry (MS). Aliquots (3 insect-hour equivalents) of Porapak Q extract were analyzed by GC-EAD (Arn et al. 1975; Gries et al. 2002), employing a Hewlett-Packard (HP) 5890 gas chromatograph fitted with a GC column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-17, DB-23, or DB-210 (J&W Scientific, Folsom, California, USA). The use of four GC columns with different retention characteristics allowed us to calculate the retention indices (RI) (Van den Dool and Kratz 1963) and intercolumn IR differentials of the antennal stimulatory compounds. This analytical method helped reveal the chemical functionality (e.g., secondary alcohol) of these compounds, and contributed to their identification (Nosil et al. unpublished data).

For GC-EAD recordings, an antenna was carefully removed from an insect's head, and its base placed into the opening of a glass capillary electrode (1.0 × 0.58 × 100 mm) (A-M Systems, Inc., Carlsborg, Washington, USA) filled with saline solution (Staddon and Everton 1980). The distal end of the antenna with its tip removed by spring micro-scissors (Fine Science Tools Inc., North Vancouver, British Columbia, Canada), was inserted into the recording capillary electrode.

Coupled GC–MS analyses of pheromone extract employed a Varian Saturn 2000 Ion Trap GC–MS fitted with the DB-5 or DB-17 column.

GC-EAD analyses of female *T. cristinae* effluvia revealed several volatiles that elicited strong responses from male antennae (Fig. 4). The volatile and antennal response profiles were very similar for HVA and PR populations. GC–MS analyses confirmed that the same components were EAD-active in HVA and PR populations. The mass spectrum and retention indices (RI) (Van den Dool and Kratz 1963) of the most EAD-active component (DB-5: RI = 1258; DB-17: RI = 1472; DB-23: RI = 1909; DB-210: RI = 1476) in both populations were strongly indicative of a secondary alcohol.

We then performed no-choice experiments using static-air olfactometers (Vet 1983, Takacs and Gries 2001) to assess whether pheromonal cues contribute to mate discrimination. These experiments provide evidence for olfactory-based discrimination independent of the molecular structure of the pheromone component(s). The olfactometers' three chambers (each 10 cm diameter × 3.5 cm high) were linearly interconnected by a glass tube (each 1 cm diameter × 2.5 cm long). A no-choice design was used because it allowed a direct comparison to the no-choice experiments used to quantify sexual isolation (Nosil et al. 2002, 2003), and it avoids confounding mating propensity with mate preference (Rolan-Alavarez and Cabarello 2000). Thus for each trial a male was tested against a single female that was from either the males own population or from the alternative population. The experiment tests whether a male exhibits no response (stays in the central release chamber), responds to the test stimulus (by approaching the female), or responds to the control stimulus (approaches the empty chamber).

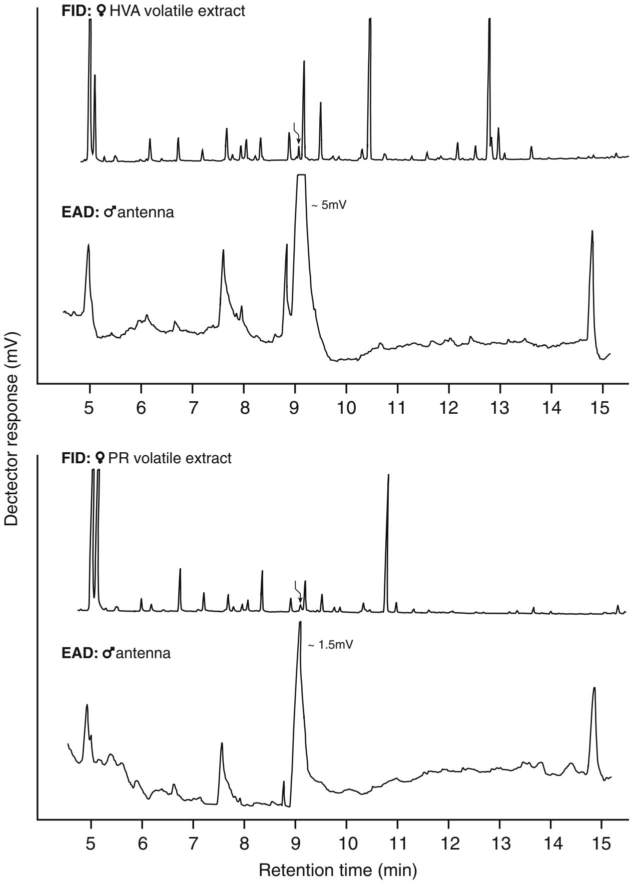

Fig. 4 Flame ionization detector (FID) and electroantennographic detector (EAD: male *Timema cristinae* antenna) responses to effluvium volatiles from female HVA (*top*) and female PR (*bottom*). Chromatography: DB-5 GC column (0.32 mm ID × 30 m); temperature program: 50°C (1 min), 10°C per min to 200°C, then 25°C per min to 280°C. Most antennally active compound marked by an arrow

The experiment proceeded in four steps. First, olfactometers were randomly assigned to locations on the laboratory bench. Second, females were randomly assigned to individual olfactometers and to one of the two side chambers. Females were confined within a glass tube covered with wire mesh to prevent physical contact of the bioassayed male with a female as the test stimulus. The control chamber contained an empty glass tube of the same type. Third, a male was released in the central chamber. Fourth, all activity of the male was recorded for two hours. Specifically, we recorded when the male moved, and which chamber it entered. We analyze the first movement of each male because multiple movements occurred very rarely (5% of trials). Between trials run every other day, olfactometers were rinsed with 70% ethanol, thoroughly washed with soap, and left to dry 24 h (i.e. experiments were run every second day). A total of 318 trials were conducted (Experiment 1: 192 trials for HVA×PR; Experiment 2: 126 trials for HVA×OUTA).

We performed three statistical analyses for each experiment, predicting larger between-population discrimination in the different host experiment (Experiment 1). The first logistic regression analysis uses data from all the trials for a particular experiment. It tests whether a male's approach towards the female (binary dependent variable—approached occurred or not) was dependent on the male population, female population or the interaction between the male and female population. We are interested in the interaction term, which tests whether the response of a male from a particular population is dependent on the population of female. We also included male color morph, male age upon capture (sexually immature versus mature), female color morph and female age upon capture as factors in the analysis. Models not including these factors yielded similar results and are not shown. Significance was assessed using likelihood-ratio tests from full and reduced models. The second analysis was the same as the first, with one modification; we excluded all the trials where a male remained in the central release chamber for the entire trial. This exclusion allowed us to test whether the results of the first analyses are confirmed when only active males are considered. The third analysis examined only those trials where a female was chosen, and tested which factors predict the time period until the female is chosen. The time until a female was chosen is continuously-distributed such that all the terms described in the other two analyses were included, but analyzed using ANOVA.

Evidence for pheromone-based discrimination

We detected evidence for olfactory-based behavioral discrimination for the population pair using different hosts, but not for the pair using the same host (Table 4 for statistics and Fig. 5 for data). Thus for the HVA × PR comparison, the probability that a female was approached was significantly greater for within-population trials than for between-population trials. This pattern occurred both when all trials were considered, and when only trials where a male entered a side chamber were considered ($p < 0.05$ in both cases, see Fig. 5A–C and Table 4). Additionally, when only the trials where a female was approached were examined, the time until a female was approached was significantly lower for within-population versus between-population trials, as expected if there was between-population discrimination based upon olfactory cues. In contrast to the results with the pair using

Table 4 Statistical analyses showing that responses of male *T. cristinae* to olfactory cues are dependent on an interaction between male population and female population (i.e. male population × female population interaction term), but only for the population pair using different hosts (i.e. Experiment #1)

Test	*n*	Model	Test-statistic	*p*-Value
Experiment #1—Populations on different hosts				
1. Was a female chosen?	192	Full	5.01	0.024
		Reduced	7.29	0.007
2. If the male left the center, was a female chosen?	54	Full	8.14	0.004
		Reduced	9.07	0.003
3. When a female was chosen, how long did it take?	30	Full	4.34	0.051
		Reduced	4.77	0.019
Experiment #2—Populations on the same host				
1. Was a female chosen?	126	Full	0.20	0.65
		Reduced	Removed	>0.10
2. If the male left the center, was a female chosen?	26	Full	0.55	0.46
		Reduced	Removed	>0.10
3. When a female was chosen, how long did it take?	15	Full	1.65	0.29
		Reduced	Removed	>0.10

Tests 1 and 2 use logistic regression analyses (test-statistic is -2LR) and test 3 uses ANOVA (test statistic is *F*-ratio). The 'full model' included all the factors examined and the interaction term, and 'reduced model' removed all terms for which $p > 0.10$ (Materials and methods for details)

different hosts, there was little or no evidence for olfactory-based discrimination in the trials using the population pair on the same hosts. For the HVA × OUTA comparison, significant differences for within-population versus between-population trials were not detected in any case (all $p > 0.25$). In fact, trends were sometimes in the direction opposite to that expected if there was between-population discrimination. For example, the time taken for a male to approach a female tended to be greater when the female was from the same population (Fig. 5D–F and Table 4).

The results suggest that sexual isolation between populations of *T. cristinae* that use different host plants involves, at least in part, pheromonal communication. Behavioral experiments revealed evidence for discrimination based upon pheromonal cues, and several candidate pheromone components in the insects' effluvium elicited antennal responses. Subtle, rather than large, differences in the insects' volatile effluvia and antennal response pattern were detected. One hypothesis is that the host forms of *T. cristinae* differ in some qualitative aspect of pheromones, such as enantiomer composition, and that discrimination is based on enantiospecific responses. The most antennally active candidate pheromone component indeed can exist as two enantiomers (Nosil et al. unpublished data) which may or may not be so for the as yet unidentified candidate pheromone components. Enantiospecific pheromone production and differential attraction to specific blends of enantiomers has been reported in other insects (Millar et al. 1990, 1991; Miller et al. 1996; Gries et al. 1999, 2003). Such enantiospecific responses have been shown to result in assortative mating (Teale et al. 1994) and partial barriers to gene flow between pheromone races (Cognato et al. 1999). Further work could examine whether the enantiomer ratio of major pheromone components differs between populations. We also do not know how strongly pheromone production in *T. cristinae* is affected by environmental (i.e. host-plant reared upon) versus genetic factors. However, sexual isolation itself is unaffected by rearing environment (Nosil et al. 2003), and pheromonal discrimination in our behavioral experiments was the same for field-caught adults and for nymphs reared to maturity in the lab in a common environment (i.e. on *Ceanothus*). Other studies of insect pheromones have detected a strong genetic basis to their composition (Seybold et al. 1995; Hager and Teale 1996).

We examined male responses to female pheromones, because sexual isolation between allopatric pairs of populations involves preference divergence of both sexes, and the male approach to females is an important component of sexual isolation (Nosil et al. 2002). It is not yet possible to determine conclusively whether divergence in pheromonal communication is linked to adaptation to different host plants, because only two population pairs have been examined. However this level of replication is highly typical of studies of the traits underlying sexual isolation (Ryan et al. 1990; Nagel and Schluter 1998; Cruz et al. 2001; Jiggins et al. 2001; Fordyce et al. 2002; Vines and Schluter 2006). Two lines of evidence are highly suggestive.

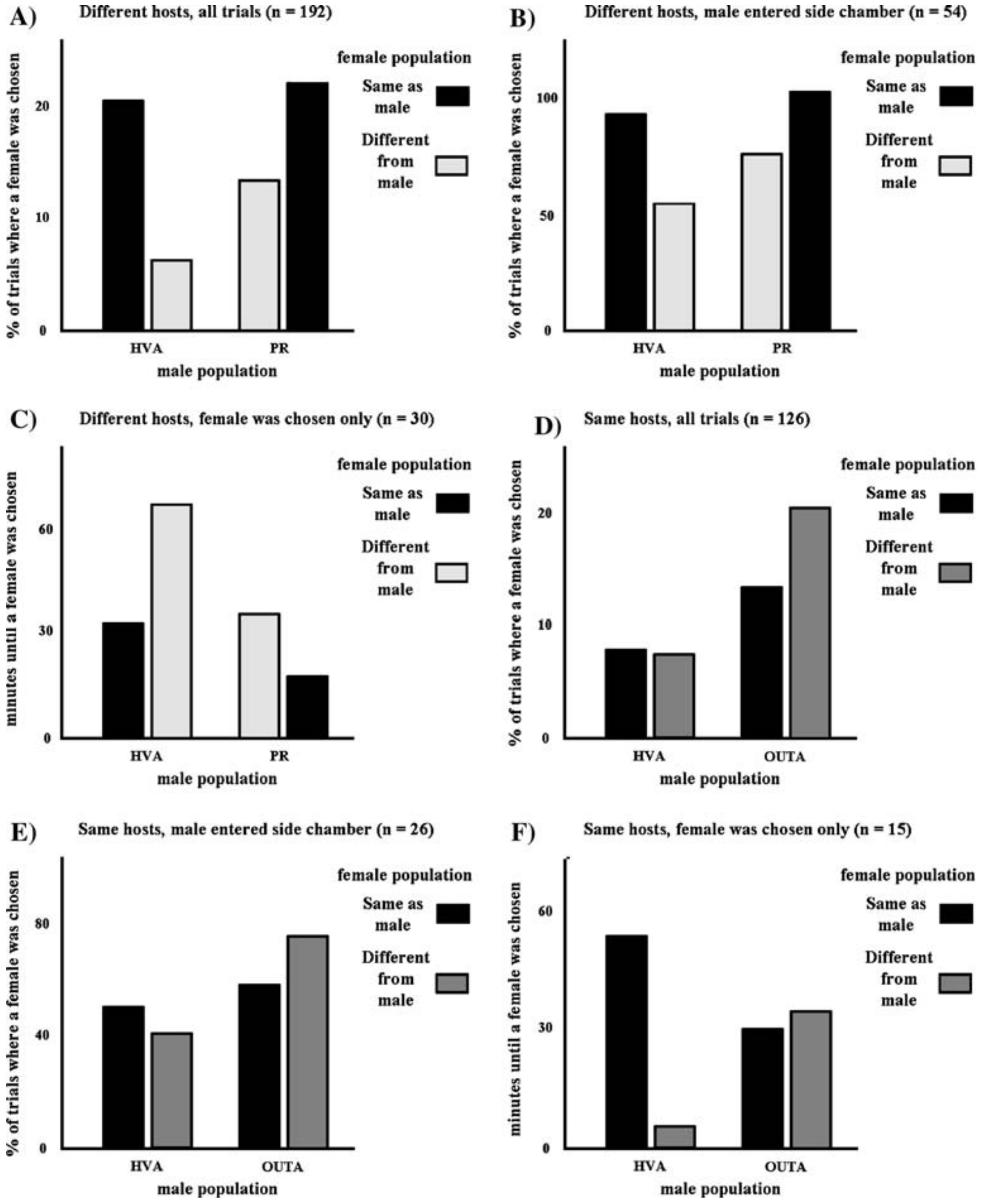

Fig. 5 Olfactometer experiments showing that male discrimination based on olfactory cues occurs only for the *T. cristinae* population pair using different hosts. Panels **A**–**C** show the results from a population pair that uses different hosts (HVA × PR), where discrimination based on olfactory cues was detected (all $p < 0.05$). Panels **D**–**F** show the results from a population pair that uses the same host (HVA × OUTA), where discrimination based on olfactory cues was not detected (all $p > 0.15$)

First, pheromonal discrimination was detected between the population pair on different hosts, but not between the pair on the same host. Second, male mating preferences are not reinforced in parapatry, such that discrimination by males based upon pheromonal cues is more likely to involve local adaptation than reinforcement (Nosil et al. 2003).

Sexual isolation due to local adaptation versus reinforcement need not involve divergence in the same traits. In *T. cristinae*, it appears that preferences of both sexes diverge due to local adaptation, with only female preferences reinforced in parapatry. Overall variation in sexual isolation could thus involve multiple traits, as has been observed in *Drosophila*. Sexual isolation between allopatric populations of *D. pseudoobscura* and *D. persimilis* involves divergence in courtship song (Williams et al. 2001), whereas genomic regions affecting reinforced mating discrimination contain genes involved in pheromonal communication (Ortiz-Barrientos et al. 2004; see also Boughman et al. 2005 for composite mating traits in stickleback). Future studies of *T. cristinae* could examine the possibility of composite mating traits, particularly because courtship behavior occurs but has not been examined.

Finally, we note that few studies of sexual isolation have examined mating preferences and also determined the traits (e.g. color-patterns, body size, pheromones, behavior; but see Boughman et al. 2005) upon which mate preferences are based. In the few cases of reinforcement where both have been examined, preferences often exhibit greater displacement than the mating traits themselves, suggesting that selection acts mostly on receiver selectivity (Hobel and Gerhardt 2003). In cases of local adaptation, interactions between natural and sexual selection are often involved in the divergence of traits and preferences (Naisbit et al. 2001; Leal and Fleishman 2004; Boughman et al. 2005). It would be of interest to further quantify geographic variation in pheromones and other potential mating traits among populations of *T. cristinae*, and to compare this variation to geographic variation in preferences. Determining that phermonal communication is involved in sexual isolation is a major step because it will allow such studies.

General discussion

Our approach to examining the contribution of different selective processes to the evolution of sexual isolation may be applied to other systems, and to other forms of preference (e.g. habitat preferences, temporal isolation). Many studies have examined the effects of one or two processes on the evolution of sexual isolation (e.g. herbivorous insects—Funk 1998; *Drosophila*—Etges 2002; flowering plants—Bradshaw and Schemske 2003; stickleback fishes—Rundle et al. 2000; McKinnon et al. 2004; butterflies—Jiggins et al. 2001; Fordyce et al. 2002; intertidal snails—Rolan-Alvarez et al. 1997, Cruz et al. 2004). Outstanding questions concern the relative importance of distinct evolutionary forces such as ecological selection, reinforcing selection and gene flow, and the traits involved. Ascertaining these factors will require detailed studies of individual systems. For example, in the cricket frog (*Acris crepitans*) environmental selection for transmission efficiency must be integral for call divergence because alternative hypotheses such as reinforcement and pleiotropic effects of body size divergence are ruled out (Ryan et al. 1990; Ryan and Wilczynski 1991).

In cases of reproductive character displacement, very few systems have examined multiple costs to hybridization or the potential role of gene flow (Servedio and Noor 2003). In *T. cristinae*, the effects of reinforcing selection are greatest under intermediate migration rates, where encounters between populations are common enough to promote reinforcement, but low enough to prevent gene flow from eroding divergence (Nosil et al. 2003; see also Noor 1995). Additionally, although *T. cristinae* females are selected to be more discriminating against males from an adjacent population, this selection has indirectly resulted in increased mating discrimination against foreign males from multiple other populations that use either host (Nosil et al. 2003). Such 'universal' effects of reinforcement may be due to females recognizing and preferring males from their own population based on a 'population-specific' trait instead of a 'host-specific' or 'species-specific' trait (Kelly and Noor 1996; Higgie et al. 2000; see also Zouros and D'Entremont 1980; Hoskin et al. 2005). If such 'universal' effects are common, then reinforcement could contribute to speciation between ecologically similar pairs of populations, between populations that are geographically separated from one another, and between conspecific populations. Notably, preference for population-specific traits makes reinforcement theoretically more likely in an island-continent scenario (Servedio 2000), perhaps explaining why reinforcement occurs in *T. cristinae* in the face of highly asymmetric gene flow.

Although the current study focused on premating isolation, it also presents some data on postmating isolation. We detected no evidence for reduced F1 hatching success in between-population versus within-population crosses. Our data do not allow us to disentangle the effects of differential fertilization (e.g. gametic isolation, Palumbi 1998; Swanson and Vacquier 2002) from differential mortality of embryos. However, we detected no differences in hatching success, suggesting that neither process occurs. An alternative explanation, which is somewhat contrived, is that fertilization success and embryonic death work in opposite directions in different types of crosses such that they exactly cancel each other out (yielding equal hatching success in all cross types). More likely, neither differential fertilization nor differential embryonic mortality occurs. A lack of intrinsic F1 hybrid inviability between sister species has certainly been observed in nature (Schluter 2000 for review; Johannesson et al. 2000; Saldamando et al. 2005), but examples where it does occur are also common (Coyne and Orr 1989, 2004).

Most work on speciation via natural selection ('ecological speciation') has not examined gametic isolation and intrinsic inviability (but see Lu and Bernatchez 1998), perhaps because these barriers can evolve via any form of selection or by genetic drift (Rundle and Nosil 2005 for review). Previous work on other systems has implicated a role for selection in both the evolution of gametic isolation and intrinsic reductions in hybrid fitness. Reproductive proteins involved in fertilization often evolve rapidly via selection (Vacquier et al. 1997; Swanson and Vacquier 2002). Likewise, the three genes causing intrinsic postzygotic isolation which have been identified exhibit a history of evolution via positive selection (*Hmr*, Barbash et al. 2004; *Nup96*, Presgraves et al. 2003; *OdsH*, Ting et al.

1998; Wu and Ting 2004 for review; Shuker et al. 2005 for a potential counterexample). However, the causes of selection (e.g. ecological or not) cannot be determined from these data alone. Gene flow and population subdivision are also predicted to affect the evolution of genetic incompatibilities (Orr and Orr 1996; Church and Taylor 2002; Gavrilets 2004). The current study examined scenarios where ecology and gene flow varied, but the overall lack of inviability precludes a strong test of the effects of either factor. Further studies are clearly warranted, because the processes driving the evolution of genetic incompatibilities remain poorly understood.

Barriers to gene flow that evolve before reproductive isolation is complete can provide particular insight into speciation, and thus the temporal order of evolution of different forms of isolation is important. A few comparative studies indicate that sexual isolation can evolve before intrinsic postmating isolation but the generality of this finding is unknown (Coyne and Orr 1989, 1997; Mendelson 2003; Ramsey et al. 2003). Every form of reproductive isolation examined previously in *T. cristinae* was detected (Table 1). Populations on different hosts are partially reproductively isolated by divergent host preferences (Nosil et al. 2006a, b), low survival of between-host migrants (Nosil 2004), sexual isolation (Nosil et al. 2002, 2003), and postmating, prezygotic incompatibilities (Nosil and Crespi 2006). The current study focused on the same populations examined in previous work such that in these populations premating isolation has evolved earlier than intrinsic hybrid egg inviability.

Speciation is often a continuous process whereby populations diverge from randomly mating units to reproductively isolated species. Theory clearly demonstrates that the evolution of sexual isolation can involve many selective processes, and our study shows how their contributions might be examined. The results show that multiple processes can act simultaneously, with a central role for ecology and geography. The host-associated forms of *T. cristinae* are unlikely to have achieved species status, as indicated by only partial barriers to gene flow at the premating level and weak mtDNA differentiation between adjacent populations on different hosts due to ongoing gene flow (Nosil et al. 2003). The host forms represent either an ongoing speciation event or population divergence that has reached equilibrium such that we have examined the early stages of the speciation process. Studies of more divergent taxa are required to test how our findings apply to the latter stages of speciation (Sandoval and Nosil 2005). The degree to which the same traits and processes are involved at different stages of speciation is poorly understood (Claridge and Morgan 1993; Ryan and Rand 1993; Mallet et al. 1998; Boake et al. 1997; Bordenstein et al. 2000; Jiggins et al. 2004). Studies examining both traits and preferences, and multiple selective processes at different stages of divergence, will likely provide the most complete picture of the entire speciation process.

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