Dimensionality of sexual isolation during reinforcement and ecological speciation in *Timema cristinae* stick insects

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ABSTRACT

Background: Speciation can involve variation in the dimensionality of population divergence (defined as variation in the number of independent ecological variables, phenotypic traits, or genes differing between populations). Recent work indicates that reproductive isolation between populations is multidimensional, but that this multidimensionality has an upper limit. A remaining question is how ecological and geographic factors affect the dimensionality of reproductive isolation. We address this question here, focusing on sexual isolation.

Methods: The data used stem from previously published mating trials from 12 populations (comprising 66 pairwise population comparisons) of *Timema cristinae* stick insects that vary in host use and geographic arrangement (allopatry versus parapatry). We estimated the dimensionality of sexual isolation by calculating independent axes of divergence that represent combinations of (unmeasured) phenotypic traits and mating preferences that determine degree of sexual isolation. We then examined how populations varied along these axes.

Results and conclusions: We found that despite the large number of population comparisons examined, only two dimensions were required to explain observed variation in sexual isolation. Furthermore, patterns of divergence illuminated ecological and geographic effects on the dimensionality of reproductive isolation. Males and females from allopatric populations were tightly clustered along one axis, consistent with sexual selection balanced by natural selection towards a single optimum. In contrast, parapatric males were widely dispersed across both axes, as would occur if reinforcement in parapatry were driving divergence in latent traits that remain constant in allopatry. Populations clustered to some extent by host plant use, although the effect was much weaker than that of geographic arrangement. We discuss our results in terms of the factors affecting speciation. In particular, if sexual isolation tends to have a strong upper limit to its dimensionality, as observed in our dataset, levels of divergence sufficient for speciation may require multiple forms of reproductive isolation.

Keywords: mate choice, natural selection, niche dimensionality, reproductive isolation, sexual selection.

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INTRODUCTION

The number of independent ecological variables, traits, or genes involved in adaptive divergence and speciation (i.e. the 'dimensionality' of divergence) has received attention in several areas of evolutionary biology (Price, 2007; for reviews, see Nosil and Harmon, 2009; Nosil et al., 2009). For example, data from laboratory evolution studies suggest that 'multifarious selection' on numerous traits or genes promotes speciation (Rice and Salt, 1988; Rice and Hostert, 1993). There is also some evidence to support this hypothesis in natural populations (Dambroski and Feder, 2007; Nosil and Sandoval, 2008; Seehausen, 2008; Seehausen et al., 2008; Lawniczak et al., 2010; Michel et al., 2010). Multifarious selection might promote speciation because differentiation in more traits results in an increased probability of divergence in key traits that affect reproductive isolation and because it can promote overall genetic divergence. Some theoretical models also support these observations (Nosil and Harmon, 2009; Nosil et al., 2009; Feder and Nosil, 2010). In this context, highly dimensional ecological niches might be important for generating new species.

Evolution along multiple axes or dimensions can be conceptualized in the adaptive landscape model, and the dimensionality of adaptive landscapes may have important consequences for our understanding of phenotypic evolution (for reviews, see Gavrilets, 1997, 2003, 2004; Schluter, 2000b). Traditional adaptive landscapes are rugged, with distinct peaks and valleys of high and low fitness, respectively (Schluter and Grant, 1984). The question of how populations can traverse valleys of low fitness and thus achieve adaptive peak shifts is a classical and ongoing problem in evolutionary biology (Schluter, 2000a; Templeton, 2008; Schluter et al., 2010). However, it has been argued that this view of landscapes as rugged, and thus resistant to peak shifts, is largely an artifact of considering low-dimensional landscapes (Gavrilets, 2003, 2004). If more dimensions are considered, landscapes will likely be 'holey' rather than rugged, with many combinations of traits or genes that accrue similarly high (but not necessarily maximal) fitness such that populations will evolve predominantly by traversing along ridges of moderate to high (but not maximal) fitness. Most support for holey landscapes is theoretical, although some data support their existence (Rhode and Cruzan, 2005; Whibley et al., 2006; for reviews, see Gavrilets, 1997, 2003, 2004).

The dimensionality of genetic variation may also constrain the paths available to adaptive evolution. While additive genetic variation may be present for many traits measured separately, strong genetic correlations among traits mean that the number of independent axes of genetic variation available to evolution may be far fewer than the number of traits (Chenoweth and Blows, 2008; Hansen and Houle, 2008; Kirkpatrick, 2009; Walsh and Blows, 2009). As in the holey adaptive landscape model, the axes along which evolution can proceed may be a lower-dimensional subspace of the full phenotypic space.

Dimensionality has also been considered in the study of sexual selection, sexual isolation, and speciation. Sexual isolation arises when between-population mating is less common than within-population mating and often contributes strongly to speciation (Coyne and Orr, 1989, 2004). Mate choice can be highly complex and multivariate, involving a wide range of sensory modalities (chemical, auditory, visual, tactile, etc.) (Jennions and Petrie, 1997; Hebets and Uetz, 1999; Blows et al., 2003; Candolin, 2003; Brooks et al., 2005; Chenoweth and Blows, 2006; Uy et al., 2009). The large number of traits involved leads to the prediction that sexual isolation results from divergence in multiple dimensions. However, a recent analysis (Hohenlohe and Arnold, 2010) suggests that the dimensionality of sexual isolation is actually much lower than the number of traits putatively involved in mate choice. That is, despite the large number of actual traits and
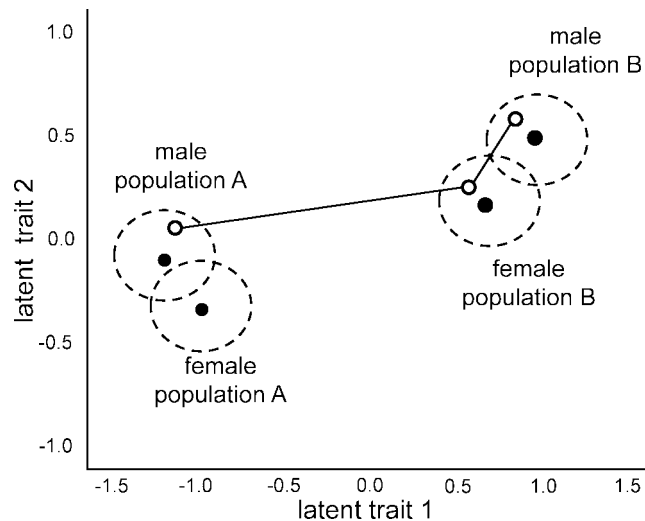
Fig. 1. Schematic representation of the mate-choice model for two populations (A and B) in two latent trait dimensions, with population means (solid circles) and within-population variation (dashed circles) for male traits and female preferences. The probability of mating between, for example, a female chosen randomly from population B and a male chosen from either the same population or population A is a function of the distance between them (solid black lines) in this phenotypic space. Modified from Hohenlohe and Arnold (2010) with permission of the National Academy of Sciences.

preferences involved in mate choice, evolution of sexual isolation appears limited to a few axes of divergence.

The technique of Hohenlohe and Arnold (2010) uses data from mating trials across groups of related populations (Fig. 1) to infer the populations' positions along 'latent trait axes', representing combinations of highly correlated traits and preferences that influence variation in sexual isolation. The number of latent trait axes required in the best-fit model gives the dimensionality of sexual isolation. Note that the underlying traits and preferences do not need to be measured, or even known, to estimate dimensionality of sexual isolation using this method. The only data required in the analysis are results of experimental mating trials. Mapping of populations along the latent trait axes allows tests of predictions from sexual selection models, such as the presence of natural selection acting on sexually selected traits. It also allows correlation of divergence along latent trait axes with other factors, such as ecology and geography, to better understand the role of these factors in generating reproductive isolation and facilitating speciation.

Here we apply the method of Hohenlohe and Arnold (2010) to published data on mating preference from 12 populations of *Timema cristinae* stick insects (i.e. 66 sets of pairwise mating trials). We find that despite the large number of populations examined, only two latent trait dimensions are required to explain observed variation in sexual isolation. Furthermore, we find a strong signal consistent with ecologically driven reinforcement, causing not just a higher degree of divergence but divergence along a novel latent trait axis, in parapatric versus allopatric population pairs. This reinforcement-driven divergence appears stronger than the association between reproductive isolation and host plant use by the insects.

METHODS AND MATERIALS

Study system

Timema are wingless, herbivorous insects inhabiting Southwestern North America (Crespi and Sandoval, 2000). The current study considers *T. cristinae*, which uses two host species (*Adenostoma fasciculatum*: Rosaceae and *Ceanothus spinosus*: Rhamnaceae) (Nosil, 2007). As in past work, a 'population' of *T. cristinae* is defined as all individuals of this species collected within a homogeneous patch of a single host species (Nosil, 2007). Patches of the two host species are often distributed adjacently and in direct geographic contact with one another. Insect populations associated with such patches are referred to as 'parapatric' (Nosil *et al.*, 2003). Other host patches are separated from patches of the alternative host, usually via regions containing unsuitable hosts. All such geographically separated patches are termed 'allopatric'.

There is strong evidence in this system that divergent host adaptation has contributed to reproductive isolation and is causing ecological speciation (Nosil *et al.*, 2003; Nosil, 2007). For example, pairs of populations using different host species exhibit much stronger reproductive isolation than similar-aged pairs of populations using the same host species (i.e. but existing in different localities). In addition, there is evidence for ecologically based reinforcement. Populations using different hosts exhibit adaptive divergence in a suite of morphological characters (Sandoval, 1994). Field experiments have demonstrated that these traits are subject to divergent selection between hosts (Sandoval, 1994; Nosil and Crespi, 2006b). Hybrids between the host ecotypes are intrinsically viable and fertile, but are often intermediate for morphological characters, and thus suffer reduced fitness on each host. This extrinsic post-mating isolation creates the opportunity for reinforcement in areas where the ecotypes are in contact. As predicted by reinforcement, a study of mating isolation revealed consistently stronger mating isolation between parapatric populations than between allopatric ones (Nosil *et al.*, 2003). A common-garden experiment in this same study demonstrated these differences were heritable.

Here, we re-analyse mating data from *T. cristinae* to examine a new issue, i.e. the dimensionality of sexual isolation. Protocols for the 3320 no-choice mating trials used in this study have been published previously (Nosil *et al.*, 2003). In brief, a pair of stick insects (one male and one female) was placed in a 10 cm petri dish and at the end of one hour, whether the male and female were copulating or not, was scored. The raw mating data are presented in Appendix 1 and characteristics of each of the study populations in Table 1. A map of the study populations is shown in Fig. 2.

Dimensionality analysis

We estimated the dimensionality of sexual isolation using procedures outlined in detail in Hohenlohe and Arnold (2010). The only data used in this analysis are the pairwise mating trials among a set of populations. Importantly, the analysis does not require any measurements or even prior knowledge of specific traits involved in sexual isolation. This potentially large number of hidden traits is modelled as a set of continuous, normally distributed traits in each sex, such that the probability of mating is highest when the trait values match between potential mates, and decreases as trait values differ between potential mates (Arnold *et al.*, 1996; Hohenlohe and Arnold, 2010). For example, a simple interpretation of these traits

Table 1. Characteristics of the study populations, for each sex

Population	Sex	Host	Geography	Latent trait 1	Latent trait 2
P	F	C	A	0.0419	−0.2221
HVC	F	C	P	0.4180	−0.1115
HVA	F	A	P	0.1624	−0.0916
MA	F	A	P	0.7223	−0.2837
LA	F	A	A	0.1130	0.2156
VPC	F	C	A	0.0359	−0.1443
VPA	F	A	P	0.0894	0.0318
OUTA	F	A	P	0.0385	−0.0106
PRC	F	C	A	0.0020	−0.2287
MBOXC	F	C	P	0.4069	−0.2874
OGC	F	C	P	0.3493	−0.1147
HA	F	A	P	0.1941	−0.0199
P	M	C	A	−0.9604	−0.0646
HVC	M	C	P	0.9035	−0.9085
HVA	M	A	P	0.1851	0.8477
MA	M	A	P	1.2519	0.2608
LA	M	A	A	−0.9633	−0.0922
VPC	M	C	A	−1.0078	−0.2161
VPA	M	A	P	−0.0495	0.9485
OUTA	M	A	P	−0.7657	0.4491
PRC	M	C	A	−1.0473	−0.1209
MBOXC	M	C	P	−0.0980	−1.1643
OGC	M	C	P	0.8295	0.9900
HA	M	A	P	−0.8516	0.3373

Note: For 'sex', M = male, F = female. For 'host', C = *Ceanothus*, A = *Adenostoma*. For geography, A = allopatric, P = parapatric. Also shown are the scores for each of the two dimensions (i.e. latent traits) in the best-fit mapping of the populations. See text for details and Fig. 2 for a map.

is as sexually selected ornaments or behaviours in one sex and absolute mating preferences for those traits in the other sex (Lande, 1981). However, the model is general to any type or combination of traits and/or preferences in males and/or females. Thus, our analysis implicitly incorporates all traits and preferences that affect sexual isolation in the experimental mating trial context, but it does not account for dimensionality of evolutionary divergence that is unrelated to sexual isolation.

We first used maximum likelihood to map points representing male and female population means in a d-dimensional space, using a technique akin to multidimensional scaling or principal coordinate analysis, in which distance between points determines mating probability (Hohenlohe and Arnold, 2010). In other words, the results of laboratory mating trials are treated as a sort of pairwise distance matrix, and points are fit in a d-dimensional space so that the distances between them match as closely as possible to the observed frequencies of mating, under the model illustrated in Fig. 1. The data for each cell of the matrix (i.e. males from one population and females from the same or another population) were k successes out of n trials (Appendix 1), so we applied the binomial model described by Hohenlohe and Arnold (2010). This assumes that each mating trial between two individuals is an independent sample from the respective populations. We conducted this mapping at
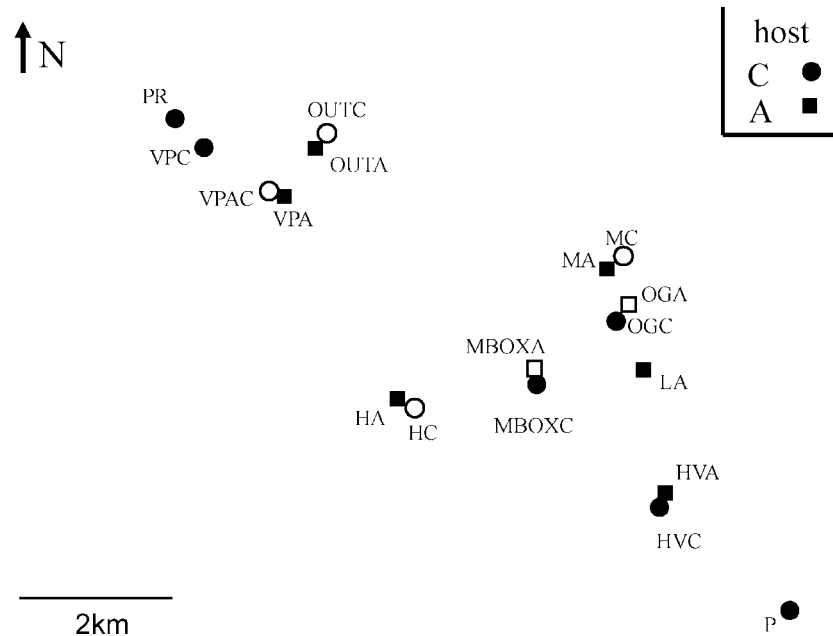
Fig. 2. Map of the study populations. Solid symbols represent populations for which mating data were obtained and open symbols populations for which data were not obtained, but which in some instances were adjacent to populations for which data were obtained. C = *Ceanothus*, A = *Adenostoma*. See Nosil *et al.* (2003) for more information concerning these populations.

values of *d* from 1 to 10. We then compared these mappings to each other to find the value of *d* at which the observed data were best explained – this is the dimensionality of sexual isolation. No single statistic is ideal for this comparison, so here we evaluated several information criterion statistics: the corrected Akaike Information Criterion [AICc (Burnham and Anderson, 1998)], the Bayesian Information Criterion [BIC (Schwarz, 1977)], and the Hannan-Quinn Information Criterion [HQC (Hannan and Quinn, 1979)]. Each method produces a score for each value of *d*, using the likelihood of the best-fit arrangement of points penalized by some combination of the number of parameters and the sample size. We evaluated AICc weights to assess the degree of confidence in a particular dimensionality. Finally, we calculated n_D, the effective number of dimensions (Kirkpatrick, 2009); the asymptotic value of this measure as populations are mapped to higher-dimensional spaces is informative to the dimensionality of the whole dataset. Software to conduct this analysis is available at http://webpages. uidaho.edu/hohenlohe/software.html.

   Axes in the best-fit multidimensional space, called 'latent traits', represent linear combinations of traits and/or preferences that influence variation in sexual isolation. For visualization and downstream analysis, we centre and rotate the latent trait axes so that latent trait 1 represents the greatest variance among populations, latent trait 2 the greatest remaining variance, and so on. The dimensionality of sexual isolation may be low as a result of few underlying traits and/or strong correlations among a large number of traits. The dimensionality estimate alone cannot distinguish between these scenarios. However, further analyses can be done on the latent trait mapping to test hypotheses about specific traits or factors affecting sexual isolation (Hohenlohe and Arnold, 2010).

Factors correlated with sexual isolation

We tested whether position in latent trait space varied according to the geographic arrangement of populations (allopatric versus parapatric) and host plant use (*Ceanothus* versus *Adenostoma*). Because we observed pronounced differences between the sexes (see Results), we analysed each sex separately. We tested for equality of variances using Levene's test and for differences in the mean using independent-sample *t*-tests. The latter were conducted assuming or not assuming equal variances, according to the outcome of the Levene's test (i.e. unequal variances were assumed in the *t*-test analyses only if the Levene's test exhibited $P < 0.05$). We tested whether latent trait axis scores were correlated between males and females from the same population using bivariate correlation. All significance values reported are two-tailed.

RESULTS

Number of dimensions

The *T. cristinae* radiation exhibited divergence along multiple latent trait dimensions (Table 2). However, there is strong evidence that the number of dimensions d is small. Both AICc and HQC assess $d = 2$ as the best-fit model, while BIC indicates $d = 1$. The 95% confidence interval using AICc weights includes only $d = 2$. However, the effective number of dimensions n_D asymptotes above a value of 3.0 when populations are mapped in higher dimensions. Further analyses below, unless otherwise described, reflect latent trait mapping at $d = 2$ (see Table 1 for latent trait scores).

Differences between the sexes

As might be expected when some level of sexual isolation exists between populations, latent trait axis scores were positively correlated between males and females from the same population, and significantly so for the first axis (latent trait axis 1: $r = 0.85$, $P < 0.001$; latent trait axis 2: $r = 0.34$, $P = 0.27$). Nonetheless, males and females tended to differ in their distribution in multidimensional latent trait space. Males were much more widely dispersed across the space than were females. Thus, males exhibited significantly more variance in both latent traits than did females (see Table 3 for parameter values and statistical details).

Effects of geographic arrangement and host plant use

The strongest and most robust result was that the geographic arrangement of a population (i.e. allopatry or parapatry) affected divergence in sexual isolation for males and, to a lesser extent, females (Fig. 3). Males from allopatric populations formed a tight cluster, separated along latent trait axis 1 from the cluster of females from the same populations. In contrast, males from parapatric populations were much more widely dispersed in latent trait space, implying that they are more widely divergent in traits and/or preferences that affect sexual isolation. These observations are often reflected in significantly different variances in latent trait values between allopatric and parapatric populations (e.g. differences were significant for both sexes in latent trait 1 but only in males for latent trait 2; see Table 3 for statistics).

Table 2. Statistics for assessing dimensionality, calculated for the full dataset, the allopatric populations alone, and the parapatric populations alone

d	lnL	#parameters	AICc	AICc weights	BIC	HQC	n_D
Full dataset							
1	−290.48	24	628.96	0.037	775.54	631.19	1.00
2	−264.74	46	622.49	0.951	902.44	625.75	1.59
3	−247.67	67	631.34	0.011	1038.56	635.56	1.67
4	−236.09	87	650.18	9.2×10^{-7}	1177.55	654.26	2.43
5	−229.47	106	677.95	8.6×10^{-13}	1318.37	680.79	2.86
6	−225.47	124	707.94	2.6×10^{-19}	1456.30	710.45	2.95
7	−223.18	141	740.35	2.4×10^{-26}	1589.54	741.44	3.17
8	−221.58	157	772.16	3.0×10^{-33}	1716.07	771.73	3.20
9	−220.78	172	803.57	4.5×10^{-40}	1836.10	801.53	3.22
10	−220.53	186	835.06	6.6×10^{-47}	1949.10	830.32	3.30
Allopatric populations							
1	−35.90	8	87.81	0.934	124.51	86.89	1.00
2	−32.56	14	93.12	0.066	157.35	91.52	1.31
3	−32.17	19	103.34	4.0×10^{-4}	189.50	100.16	2.08
Parapatric populations							
1	−119.59	16	271.18	0.139	352.6095541	270.52	1.00
2	−103.27	30	267.54	0.861	419.212751	265.29	1.64
3	−97.24	43	283.49	3.0×10^{-4}	499.3231627	278.70	2.49
4	−93.17	55	301.35	3.9×10^{-8}	576.2568539	294.07	2.70
5	−91.66	66	322.32	1.1×10^{-12}	651.2077675	312.58	2.90
6	−91.34	76	344.68	1.5×10^{-17}	721.4632609	331.53	2.88
7	−91.27	85	365.55	4.5×10^{-22}	785.1331148	349.03	2.90

Note: For each number of dimensions (d), we show the log likelihood (lnL) of the best-fit mapping, number of parameters, information criterion statistics (see text for details), and Kirkpatrick's (2009) effective dimensionality (n_D).

Despite the fact that allopatric populations are arrayed mostly along latent trait axis 1, the within-sex difference in variance between allopatric and parapatric groups is larger on axis 1 and smaller on axis 2 (Fig. 3 and Table 3).

The observations from latent trait mapping of the entire dataset are also borne out by separate dimensionality analyses of the two groups of populations (Table 2). The best-fit dimensionality of the allopatric populations considered separately is $d = 1$, while that of the parapatric populations is $d = 2$. In other words, the allopatric populations are spread along one axis of divergence in reproductive isolation traits, while the parapatric populations are spread along two axes of divergence, suggesting divergence in traits or linear combinations of traits in parapatric populations that are not divergent in allopatry.

The effect of host plant use on patterns of divergence was much less noticeable. Differences in latent trait scores only approached rather than achieved statistical significance and only did so for latent trait 2 (where $P = 0.069$ and 0.054 in males and females, respectively; see Table 3 for details).

Table 3. Results for tests of whether means and variances along the two latent trait axes in the best-fit mapping of the populations differ according to three factors: (1) the geographic arrangement of populations (allopatry versus parapatry, tested separately in each sex), (2) host plant use (*Ceanothus* versus *Adenostoma*, tested separately in each sex), and (3) sex across the whole dataset

Factor 1	Mean	Variance	Factor 2	Mean	Variance	F-value	P-value	t-value	d.f.	P-value
Latent trait 1										
Allopatry (M)	−0.995	0.002	Parapatry (M)	0.176	0.598	**7.98**	**0.018**	**4.27**	**7.08**	**0.004**
Allopatry (F)	0.048	0.002	Parapatry (F)	0.298	0.050	**5.43**	**0.042**	**3.03**	**8.13**	**0.016**
Ceanothus (M)	−0.230	0.846	*Adenostoma* (M)	−0.199	0.721	0.24	0.634	−0.06	10	0.952
Ceanothus (F)	0.209	0.041	*Adenostoma* (F)	0.220	0.064	0.04	0.838	−0.08	10	0.935
Males	−0.215	0.713	Females	0.215	0.047	**21.65**	**<0.001**	1.70	12.46	0.113
Latent trait 2										
Allopatry (M)	−0.124	0.004	Parapatry (M)	0.220	0.682	**5.57**	**0.040**	1.17	7.18	0.280
Allopatry (F)	−0.095	0.044	Parapatry (F)	−0.111	0.014	1.01	0.234	−0.17	10	0.867
Ceanothus (M)	−0.247	0.572	*Adenostoma* (M)	0.459	0.150	1.14	0.311	−2.04	10	0.069
Ceanothus (F)	−0.185	0.005	*Adenostoma* (F)	−0.026	0.027	0.96	0.350	−2.18	10	0.054
Males	0.106	0.464	Females	−0.106	0.021	**13.35**	**0.001**	−1.05	12.01	0.314

Note: The *F*-value and its significance stem from Levene's test for equality of variances. The *t*-value and its significance stem from independent sample *t*-tests. M = males, F = females. Significant results are given in **bold** font.
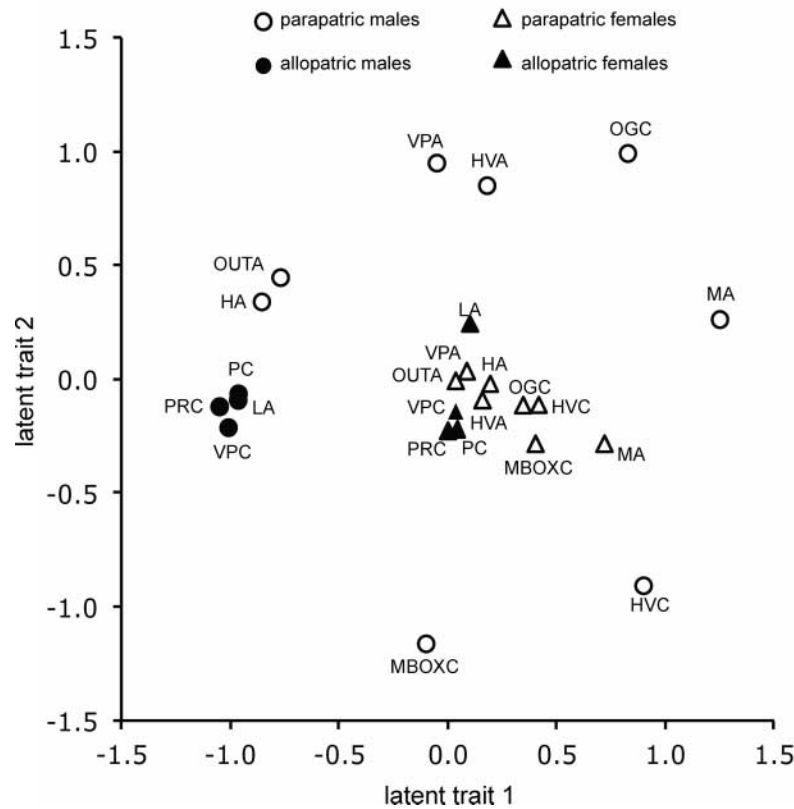
Fig. 3. Scores for the first two latent trait axes ('dimensions') for male and female *T. cristinae* from allopatric versus parapatric populations. Host use is also designated by the last letter of each population abbreviation (A = *Adenostoma* and C = *Ceanothus*). Note that allopatric males are clustered, and parapatric males widely distributed across dimensional space. There is also some separation in females according to host plant use.

DISCUSSION

We examined the dimensionality of sexual isolation in *T. cristinae* stick insects. We found that only two dimensions were required to explain variation in sexual isolation among 12 populations. This result is consistent with a past study examining nine different radiations (Hohenlohe and Arnold, 2010). The collective results indicate that although sexual isolation does appear multidimensional, as predicted by multi-modal signalling, there is an upper limit to dimensionality. Furthermore, several factors affected the position of populations in multidimensional latent trait space, the foremost being the geographic arrangement of populations. The findings have implications for three main issues in evolutionary biology: (1) factors driving sexual isolation, (2) the specific traits and preferences involved in sexual isolation, and (3) how geographically widespread the effects of reinforcement are. We discuss each of these issues in turn.

Sexual isolation in *Timema* stick insects

The causes of sexual isolation among populations of *T. cristinae* have been reviewed in detail elsewhere (Nosil *et al.*, 2007). In general, sexual isolation appears to evolve in part due to adaptation to different host plant species, but is accentuated in parapatry versus allopatry (Nosil *et al.*, 2003). Two factors may produce this accentuated sexual isolation in parapatry: reinforcement and direct selection on mating preference. As noted already, selection against unfit hybrids can result in the evolution of sexual isolation via the process of 'reinforcement' (Dobzhansky, 1937, 1951). 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) (Kirkpatrick and Ravigne, 2002). Reinforcement is likely to occur in *T. cristinae* because hybrids between the host ecotypes are intrinsically viable and fertile, but are often intermediate for morphological characters, and thus suffer reduced fitness on each host. This extrinsic post-mating isolation creates the opportunity for reinforcement in areas where the ecotypes are in contact (Sandoval, 1994; Nosil and Crespi, 2006b).

However, costs to hybridization that do not involve hybrids can also drive divergence in mate preference. For example, hybridization can reduce the survival or fertility of parental individuals themselves, favouring the evolution of sexual isolation (Servedio, 2001; Albert and Schluter, 2004). This is highly possible in *T. cristinae*, as individuals pairing and mating with the alternative ecotype could suffer higher rates of visual predation themselves (Nosil *et al.*, 2007). Moreover, very weak associations between morphological characters affecting hybrid fitness and mate choice within populations have been documented (Nosil *et al.*, 2007) and this should constrain reinforcement.

In the current study, parapatry corresponded to divergence along a latent trait axis of traits and preferences not observed in allopatric populations. This accentuated divergence in parapatry may result from some combination of reinforcement and direct selection on hybridization, and further work is required to test the relative importance of these two factors. We found that direct effects of host plant use on divergence were much weaker than those of parapatry, and only approached statistical significance along the second latent trait axis. These results appear to reject the hypothesis that sexual isolation is driven primarily as a byproduct of selection associated with host plant use acting on traits involved in mate choice. Rather, host plant use is critical in driving the ecological divergence that creates the opportunity for reinforcement, but it appears that subsequent reinforcement and/or direct selection against hybridization are then primarily responsible for divergence in mating preferences and sexual isolation. Further studies examining the effects of multiple ecological and geographic factors on sexual isolation are warranted (e.g. Schwartz *et al.*, 2010).

Traits and preferences involved in sexual isolation

The method employed here does not test which phenotypic traits contribute to sexual isolation. Some data on this exist from past work. For example, mate choice is clearly not based upon morphological characters, either general body morphology or external genitalia (Nosil and Crespi, 2004; Arbuthnott *et al.*, 2010). Although behavioural courtship may be necessary for successful copulation, it also does not appear to contribute directly to sexual isolation (Arbuthnott and Crespi, 2009). There is some evidence that sexual isolation is based upon chemical communities (i.e. pheromones and cuticular hydrocarbons), but much work remains to be done to fully understand the traits underlying mate choice in this species (Nosil *et al.*, 2007).

In particular, further studies should examine whether sexual isolation in parapatry involves traits or behaviours not involved in mating preference between allopatric populations, as suggested by our latent trait mapping. One approach is simply to correlate latent trait values with empirical measurements of candidate traits or preferences across the populations studied; this approach has provided preliminary indications of the traits and behaviours involved in sexual isolation in *Desmognathus* salamanders and cichlid fishes (Hohenlohe and Arnold, 2010).

A major unresolved question is which sex is actually choosing during mate choice in *T. cristinae*. Several lines of evidence implicate male mate choice in *T. cristinae*. First, pheromone analyses and behavioural experiments have shown that the initial attraction of males to females is likely based upon chemical communication, with males preferring to move towards females from the same host ecotype in the absence of cues other than olfactory ones (Nosil *et al.*, 2007). Second, males selectively courted females of the same host type in some (but not all) experiments to date (Arbuthnott and Crespi, 2009). Because mate discrimination occurs after pairing but prior to courtship in *T. cristinae*, this provides evidence of a role for male mate choice in reproductive isolation between host types in *T. cristinae*, or for some male–female interaction that affects male courtship propensity. *Timema* have at least three characteristics that could promote male choice of mates (Arbuthnott and Crespi, 2009): long copulation times (3–5 h), during which males are unable to search for other potential mates or feed; a long period of post-copulatory mate guarding (1–4 days), which represents another significant time investment; and decreased fecundity of females mated with opposite host type individuals (Nosil and Crespi, 2006a; Nosil, 2007).

In our analysis, latent traits may represent any combination of traits and/or preferences in both males and females; the underlying model is agnostic on this point (Hohenlohe and Arnold, 2010). Many theoretical models assume female preference acting on male traits (Mead and Arnold, 2004). Under this assumption, stabilizing selection on male traits constrains divergence of their traits, whereas mean preferences of females can be more divergent (Lande, 1981; Mead and Arnold, 2004). This is predicted to result in less dispersion of males in latent trait space, and indeed this was the case in seven of nine radiations analysed by Hohenlohe and Arnold (2010). In strong contrast, we observed the opposite result here, with male values being significantly more widely distributed in latent trait space than female values (Fig. 3; Table 3). This was driven primarily by the wide dispersion of males from parapatric populations in latent trait space, as discussed above. This result is consistent with the hypothesis that sexual isolation in *Timema* is primarily a result of male preference acting on female traits, with natural selection on female traits constraining divergence of females along latent trait axes relative to males. However, many other factors may also play a role, such as direct costs to mating preferences (Pomiankowski *et al.*, 1991; Mead and Arnold, 2004; Uyeda *et al.*, 2009). Most theoretical modelling of interactions between sexual selection, natural selection, and preference costs has focused on equilibrium behaviour within a single population (Mead and Arnold, 2004). Further work is needed to establish predictions about relative divergence of males and females among populations, and it is likely that multiple combinations of factors could produce the greater level of divergence among males observed here.

Support for the 'cascade reinforcement' hypothesis

There are many examples of greater sexual isolation between hybridizing populations relative to allopatric ones (for reviews, see Servedio and Noor, 2003; Ortiz-Barrientos *et al.*, 2009). This pattern

of 'reproductive character displacement' is indicative of reinforcement (Rundle and Schluter, 1998; Nosil *et al.*, 2003; Pfennig and Rice, 2007; Pfennig *et al.*, 2007). Reinforcement is generally invoked to explain levels of sexual isolation between a specific pair of hybridizing species. However, reinforcement might have much more widespread effects because mating discrimination evolving in response to maladaptive hybridization between species might induce further effects on mate choice within species (for a review, see Ortiz-Barrientos *et al.*, 2009). For example, reinforcement between species has been documented to result in females more readily rejecting males of their own species (Pfennig and Pfennig, 2005; Higgie and Blows, 2007; Pfennig, 2007).

The effects of reinforcement between species on the evolution of premating isolation among populations within species have rarely been explored. An exception concerns different species of frogs from the genus *Litoria.* These species appear to have speciated by reinforcement, but also, because of increases in strength of mating preferences due to reinforcement, they have evolved divergent mate preferences between populations within species (Hoskin *et al.*, 2005). A similar pattern has been reported in another frog (Lemmon *et al.*, 2004; Lemmon, 2009) and in past studies of reinforcement of *T. cristinae* (Nosil *et al.*, 2003, 2007). In *T. cristinae*, although individuals are selected to be more discriminating against individuals from a single adjacent population that is adapted to feeding on a different host plant species, individuals from parapatric populations exhibit increased mating discrimination against individuals from multiple other populations, including those from other populations that use the same host. This effect was confirmed and illustrated by our analysis: parapatry corresponds to much greater divergence in latent traits, including much greater divergence among populations using the same host plant.

Ortiz-Barrientos *et al.* (2009) proposed that this mechanism, by which the effects of reinforcement within a particular taxon pair (e.g. a specific species pair in sympatry) cascade to incidentally result in reproductive isolation among other taxon pairs (e.g. among populations within the species in the aforementioned pair), be termed the 'cascade reinforcement' hypothesis. Most generally, this hypothesis predicts that the evolution of pre-zygotic isolation between sympatric species can lead to the evolution of reproductive isolation within species. The hypothesis may often involve sexual selection within species. Such cascade effects of reinforcement on the evolution of reproductive isolation within species may be due to females recognizing and preferring males from their own population based on a 'population-specific' trait, instead of an 'ecology-specific' or 'species-specific' trait (Zouros and D'Entremont, 1980; Higgie *et al.*, 2000; Hoskin *et al.*, 2005; Higgie and Blows, 2007). Furthermore, cascading reinforcement may depend on specific aspects of the mechanism of mate choice, which may vary among and even within species. For example, Schwartz *et al.* (2010) found that female guppies from low-predation populations discriminate against males from all high-predation populations, regardless of geography, while high-predation females only discriminate against males from neighbouring low-predation populations. If cascade effects are common, then reinforcement could contribute to speciation between ecologically similar pairs of populations and between populations that are geographically separated from one another (Pfennig and Ryan, 2006). In all these contexts, geographic contact promotes, rather than constrains, speciation. The types of dimensionality analyses presented here might prove useful for further testing the cascade reinforcement hypothesis in future studies.

CONCLUSIONS AND FUTURE WORK

Here we applied a recently developed method to examine the dimensionality of sexual isolation in a species of stick insect. The results are consistent with divergence in just a few dimensions, but with a role for reinforcement in driving sexual isolation and the diversification of mating traits and preferences. A number of issues might be usefully examined in future work. In the *Timema* system specifically, it will be of interest to determine which traits are involved in sexual isolation, and to test the extent to which sexual isolation involves male versus female mate choice (or both). Additionally, recent work has used genome-wide data from 86,130 single nucleotide polymorphisms (SNPs) to examine the genomic consequences of different speciation processes (Nosil *et al.*, 2012). The results documented an excess of highly differentiated 'outlier loci' in parapatric populations, and future studies further integrating experimental, ecological, and genomic studies will likely yield new insight into the speciation process in the *Timema* system.

Most generally, the methodology used here has thus far only been applied to a single form of reproductive isolation, i.e. sexual isolation. The general approach might be applied to matrices of other forms of reproductive isolation, such as habitat preferences or hybrid unfitness. Such analyses might help determine how different forms of reproductive isolation diverge during speciation and thus have the potential to increase our understanding of the factors driving versus constraining the formation of species. In particular, if sexual isolation tends to have a strong upper limit to its dimensionality, levels of divergence sufficient for speciation may require multiple forms of reproductive isolation.

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APPENDIX 1

Mating data between 66 paired populations (Pop) of *Timema cristinae*. The total sample size (number of mating trials) is denoted by *n*. N refers to the number of mating trials for specific combinations of males and females (e.g. N11 = male from population 1, female from population 1; N12 = male from population 1, female from population 2, etc.). C refers to number of copulating pairs (e.g. C11 is the number of copulating pairs when males are from population 1 and females are from population 1)

Pop1 × Pop2	Pair	*n*	N11	N12	N21	N22	C11	C12	C21	C22
P × HVC	1	154	110	9	24	11	38	1	7	4
P × HVA	2	287	110	43	51	83	38	9	19	33
P × MA	3	145	110	5	14	16	38	0	3	10
P × LA	4	270	110	33	44	83	38	9	19	33
P × VPC	5	292	110	45	56	81	38	17	20	24
P × VPA	6	179	110	19	21	29	38	6	4	13
P × OUTA	7	191	110	20	25	36	38	8	8	14
P × PRC	8	213	110	28	31	44	38	12	10	13
P × MBOXC	9	190	110	20	28	32	38	3	11	12
P × OGA	10	174	110	18	20	26	38	4	2	5
P × HA	11	260	110	35	60	55	38	12	16	11
HVC × HVA	12	118	11	17	7	83	4	3	3	33
HVC × MA	13	36	11	4	5	16	4	4	2	10
HVC × LA	14	112	11	10	8	83	4	2	2	23
HVC × VPC	15	115	11	16	7	81	4	6	1	24
HVC × VPA	16	51	11	5	6	29	4	1	1	13
HVC × OUTA	17	68	11	11	10	36	4	0	2	14
HVC × PRC	18	74	11	13	6	44	4	6	0	13
HVC × MBOXC	19	64	11	10	11	32	4	5	3	12
HVC × OGC	20	48	11	7	4	26	4	2	1	5
HVC × HA	21	89	11	14	9	55	4	4	1	11
HVA × MA	22	118	83	5	14	16	33	1	3	10
HVA × LA	23	221	83	25	30	83	33	17	7	23
HVA × VPC	24	256	83	49	43	81	33	15	6	24
HVA × VPA	25	159	83	22	25	29	33	8	8	13
HVA × OUTA	26	174	83	29	26	36	33	14	7	14
HVA × PRC	27	165	93	19	19	44	33	8	7	13
HVA × MBOXC	28	165	83	25	25	32	33	6	10	12
HVA × OGC	29	150	83	20	21	26	33	8	5	5
HVA × HA	30	192	83	20	34	55	33	11	16	11
MA × LA	31	117	16	10	8	83	10	2	2	23
MA × VPC	32	124	16	22	5	81	10	5	0	24
MA × VPA	33	69	16	20	4	29	10	4	1	13
MA × OUTA	34	79	16	18	9	36	10	8	1	14
MA × PRC	35	86	16	22	4	44	10	2	0	13
MA × MBOXC	36	72	16	19	5	32	10	5	1	12
MA × OGC	37	68	16	21	5	26	10	9	1	5
MA × HA	38	80	16	4	5	55	10	0	0	11
LA × VPC	39	228	83	36	28	81	23	13	9	24
LA × VPA	40	158	83	27	19	29	23	9	11	13
LA × OUTA	41	169	83	31	19	36	23	12	8	14

APPENDIX 1 – *Continued*

Pop1 × Pop2	Pair	*n*	N11	N12	N21	N22	C11	C12	C21	C22
LA × PRC	42	195	83	39	29	44	23	11	7	13
LA × MBOXC	43	142	83	16	11	32	23	1	2	12
LA × OGC	44	141	83	21	11	26	23	5	5	5
LA × HA	45	198	83	28	32	55	23	7	12	11
VPC × VPA	46	163	81	22	31	29	24	5	8	13
VPC × OUTA	47	164	81	21	26	36	24	8	14	14
VPC × PRC	48	164	81	20	19	44	24	13	6	13
VPC × MBOXC	49	166	81	18	35	32	24	2	11	12
VPC × OGC	50	136	81	12	17	26	24	1	3	5
VPC × HA	51	183	81	18	29	55	24	4	12	11
VPA × OUTA	52	110	29	27	18	36	13	11	8	14
VPA × PRC	53	121	29	25	23	44	13	8	6	13
VPA × MBOXC	54	102	29	21	20	32	13	4	5	12
VPA × OGC	55	93	29	20	18	26	13	5	7	5
VPA × HA	56	127	29	19	24	55	13	7	10	11
OUTA × PRC	57	127	36	25	22	44	14	7	5	13
OUTA × MBOXC	58	116	36	23	25	32	14	2	8	12
OUTA × OGC	59	112	36	25	25	26	14	4	3	5
OUTA × HA	60	135	36	20	24	55	14	8	11	11
PRC × MBOXC	61	108	44	9	23	32	13	1	8	12
PRC × OGC	62	106	44	12	24	26	13	3	1	5
PRC × HA	63	159	44	27	33	55	13	5	11	11
MBOXC × OGC	64	110	32	26	26	26	12	6	6	5
MBOXC × HA	65	118	32	14	17	55	12	2	4	11
OGC × HA	66	104	26	8	15	55	5	0	2	11