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DOES GENE FLOW CONSTRAIN ADAPTIVE DIVERGENCE OR VICE VERSA? A TEST USING ECOMORPHOLOGY AND SEXUAL ISOLATION IN *TIMEMA CRISTINAE* WALKING-STICKS

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Abstract.—Population differentiation often reflects a balance between divergent natural selection and the opportunity for homogenizing gene flow to erode the effects of selection. However, during ecological speciation, trait divergence results in reproductive isolation and becomes a cause, rather than a consequence, of reductions in gene flow. To assess both the causes and the reproductive consequences of morphological differentiation, we examined morphological divergence and sexual isolation among 17 populations of *Timema cristinae* walking-sticks. Individuals from populations adapted to using *Adenostoma* as a host plant tended to exhibit smaller overall body size, wide heads, and short legs relative to individuals using *Ceonothus* as a host. However, there was also significant variation in morphology among populations within host-plant species. Mean trait values for each single population could be reliably predicted based upon host-plant used and the potential for homogenizing gene flow, inferred from the size of the neighboring population using the alternate host and mitochondrial DNA estimates of gene flow. Morphology did not influence the probability of copulation in between-population mating trials. Thus, morphological divergence is facilitated by reductions in gene flow, but does not cause reductions in gene flow via the evolution of sexual isolation. Combined with rearing data indicating that size and shape have a partial genetic basis, evidence for parallel origins of the host-associated forms, and inferences from functional morphology, these results indicate that morphological divergence in *T. cristinae* reflects a balance between the effects of host-specific natural selection and gene flow. Our findings illustrate how data on mating preferences can help determine the causal associations between trait divergence and levels of gene flow.

Key words.—Geographic variation, host-specific selection, migration, reproductive isolation, speciation, trait divergence, walking-sticks.

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Both natural selection and gene flow can influence the degree of population differentiation observed in nature (Endler 1977). When populations use different habitats, divergent natural selection can cause differentiation in ecologically important characters (for review, see Schluter 2000). Conversely, gene flow between divergent populations acts as a homogenizing force, eroding population differentiation (Slatkin 1987). Determining the degree to which selection and gene flow affect population divergence has received renewed theoretical and empirical attention (Crespi 2000; Schluter 2000; Hendry et al. 2001, 2002; Lenormand 2002; Saint-Laurent et al. 2003). In particular, inverse associations between levels of gene flow between populations and the degree of adaptive differentiation in morphological or behavioral traits have been reported in a wide range of taxa, including fish (Lu and Bernatchez 1999; Hendry et al. 2002), amphibians (Storfer and Sih 1998; Storfer et al. 1999), birds (Dhondt et al. 1990; Smith et al. 1997), reptiles (King and Lawson 1995), insects (Sandoval 1994a; Ross and Keller 1995), and arachnids (Riechert 1993; Riechert and Hall 2000; Riechert et al. 2001).

Levels of gene flow often reflect geographic separation and the degree of dispersal between populations, with greater gene flow resulting in reduced trait divergence. However, levels of gene flow can also indicate the degree of reproductive isolation between populations, independent of any geographic barriers to gene flow. Indeed, cause and effect are reversed if trait divergence causes reproductive isolation, thus lowering gene flow between diverging taxa (i.e., ecological speciation; Schluter 1998; Lu and Bernatchez 1999). Thus, associations between trait divergence and reductions in gene flow can arise via two nonexclusive, but opposing, mechanisms. As noted by Hendry et al. (2001, 2002), these two processes could generate a positive feedback loop whereby low gene flow allows adaptive divergence, which in turn further reduces gene flow by increasing reproductive isolation. Despite the appeal of linking trait divergence to the evolution of reproductive isolation (e.g., Tregenza et al. 2000a,b), studies of selection–gene flow balance generally have not elucidated the causal associations between trait divergence, gene flow, and reproductive isolation. For example, divergence in traits that act as proximate mating cues will cause reductions in gene flow via the evolution of sexual isolation. Likewise, when hybrids exhibit intermediate trait values and an intermediate niche is unavailable, divergence in traits important for foraging or antipredator defense can result in ecologically dependent postmating isolation (Rundle and Whitlock 2001; Rundle 2002). Without information on whether the traits studied influence reproductive isolation, it is difficult to infer whether trait divergence is a cause or consequence of reductions in gene flow, or both.

In this study, we used a combination of morphological, molecular, and behavioral data to analyze the causes and the reproductive consequences of geographic variation in morphology in a phytophagous insect. Because this study system has been the subject of related work on divergent natural selection and speciation (Nosil et al. 2002, 2003), it allows us to draw parallels between the causes of trait divergence and the degree of reproductive isolation. Moreover, we assess

whether morphological divergence causes the evolution of premating isolation, providing a direct test of whether morphological divergence is more likely to be a cause or an effect of reductions in gene flow.

Timema walking-sticks are wingless, phytophagous insects that inhabit the chaparral of California, other areas of the western United States, and northern Mexico (Vickery 1993; Crespi and Sandoval 2000). *Timema cristinae* exhibits two genetically determined color-pattern morphs, with an unstriped morph more common on *Ceonothus spinosus* (Rhamnaceae) and a striped morph more common on *Adenostoma fasciculatum* (Rosaceae; Sandoval 1993, 1994a,b). These two host-plant species are very structurally different, with *Ceonothus* plants being relatively large, treelike, and broad leaved and *Adenostoma* plants being small, bushlike, and exhibiting thin, needlelike leaves. *Timema cristinae* is heavily preyed upon by visual predators such as lizards and birds, and each morph is more cryptic on the host plant on which it is more common (Sandoval 1994a,b). Patches of these different hosts grow in a mosaic patchwork and local color-pattern morph frequencies in *T. cristinae* are determined by a gene flow–selection balance between patches exhibiting the different selective regimes (Sandoval 1994a).

A balance between natural selection and gene flow also affects the evolution of reproductive isolation in *T. cristinae*. Levels of sexual isolation are greater between pairs of populations using different host plants than between similar-aged populations using the same host, where age refers to time since divergence as estimated using mitochondrial and nuclear DNA sequence divergence (Nosil et al. 2002). Moreover, sexual isolation has been enhanced in geographic areas where populations using different hosts interbreed and produce offspring with reduced fitness (i.e., reinforcement; Nosil et al. 2003). High levels of gene flow counteract the effects of host-adaptation and reinforcement on the evolution of reproductive isolation. Thus, the magnitude of reproductive isolation between populations is greatest when migration rates between populations adapted to alternate host plants are sufficiently high to facilitate reinforcement, but low enough that gene flow does not erode adaptive divergence in mate choice (Nosil et al. 2003).

In this study, we test two hypotheses for the causes of geographic variation in morphology among populations of *T. cristinae*. First, if host-specific natural selection is a primary cause of morphological differentiation, then populations using different host plants should exhibit greater differentiation than populations using the same host plant, and the traits examined should have a genetic basis. However, gene flow into a population from an adjacent population using the alternate host may cause the study population to become more similar to other populations using the alternate host and more differentiated from other populations using the same host that are not incurring gene flow. Under this type of a scenario, morphological differentiation evolves under a balance between natural selection and gene flow, with gene flow acting as both a homogenizing and a diversifying force, depending on its context (Slatkin 1987; Ross and Keller 1995).

Second, if divergence in body size and shape has directly driven a reduction in gene flow, via the evolution of sexual isolation, then such morphological traits should influence the probability of copulation in between-population mating trials. This latter hypothesis is of particular interest because color-pattern has diverged among populations of this species (Sandoval 1994a), but does not influence the probability of copulation in between-population mating trials (Nosil et al. 2002). Thus, the sexual isolation that has evolved between populations adapted to alternate hosts is independent of color-pattern (although color-pattern might influence within-population mate choice; Nosil et al. 2002) and the traits causing the substantive levels of sexual isolation observed among populations of this species have yet to be determined. Collectively, our results provide new insights into the causal associations between trait divergence, gene flow, and the evolution of reproductive isolation.

Materials and Methods

Study Sites, Collection, and Rearing

First-instar *T. cristinae* were collected from 17 study sites in the Santa Ynez Mountains, California, in February 2001 and 2002 using sweep nets. Other species of *Timema* do not live in sympatry with populations from these sites. Patches of the two host plant species used by *T. cristinae* are usually distributed in parapatric patches of varying size, forming a mosaic at the scale of a mountain slope. However, some host patches are geographically separated from all other host patches by regions lacking suitable hosts. We define a population of walking-sticks as all of the insects collected within a homogenous patch of a single host-plant species. Parapatric insect populations are in contact with a population of insects adapted to the alternative host (i.e., they have a neighboring population), whereas allopatric populations are separated from all other populations adapted to the alternative host by distances greater than 50 times the per generation gene flow distance (12 m is the maximum per generation dispersal distance and allopatric populations were separated from populations of the alternate host by 1–3 km). Parapatric sites were chosen such that each population had only one neighboring population of the alternate host. Individuals were collected from nine populations that use *C. spinosus* as a host and from eight populations that use *A. fasciculatum* as a host (Table 1).

Walking-sticks were maintained in glass jars at the University of California at Santa Barbara (20°C) with 10–15 individuals per jar. Individuals from different populations and the sexes were kept separate. Animals were reared to maturity (4–6 weeks of rearing) on the foliage of either their native host plant or the alternative host plant and then preserved in 80% ethanol.

Morphometrics

Seven linear measurements were taken on 1004 walking sticks (n = 497 males, 507 females); these measures included head width, left hindleg femur length, left hindleg tarsal length, abdominal length, genital length on females and genital width on males, length of the subgenital plate, and thorax width. Measurements were taken with a digital micrometer under a binocular microscope at 10–40 × magnification. Each trait was measured twice, and measurement error was low

TABLE 1. Numbers of male and female walking-stick insects measured from each of the 17 study sites (C, *Ceonothus* sites; A, *Adenostoma* sites). Also shown for each study population is the relative size (geographic area) of the neighboring patch of the alternative host plant (zero for allopatric populations) and the proportion of individuals reared on the alternative (vs. native) host plant. For cases where the population used in the current study was also examined in Sandoval (1994a), the number in brackets beside the population name refers to the site number in Sandoval (1994a).

Population	Host	Relative size of neighbor	*N* (males)	Percent reared on alternate host	*N* (females)	Percent reared on alternate host
P (17)	C	0	58	40	78	44
HVC	C	0.66	15	0	10	0
HVA (15)	A	0.34	32	6	35	20
M (8)	A	0.39	38	0	25	0
L (13)	A	0	43	35	61	44
VPC	C	0	62	53	57	53
VPA (2)	A	0.94	26	0	28	0
OUTA	A	0.67	27	0	28	0
PR (1)	C	0	34	15	48	19
MBOXC (10)	C	0.95	31	0	26	0
OGC (12)	C	0.99	25	0	30	0
H (5)	A	0.08	52	33	48	25
MBOXA (10)	A	0.05	4	0	3	0
OGA (12)	A	0.01	15	0	7	0
SC	C	0	17	0	7	0
OUTC	C	0.33	13	0	10	0
PE	C	0	5	0	6	0

for all traits (all repeatabilities > 0.90, $P < 0.001$). The average of the two measurements was used in all statistical analyses. We also recorded the color-pattern of each individual (striped or unstriped; Sandoval 1994a,b for details). To reduce potential bias, all measurements were done without reference to population of origin and were carried out by one individual (P. Nosil).

DNA Sequencing

Migration rates between populations using different host-plant species were inferred using 107 previously published mitochondrial DNA (cytochrome oxidase I) sequences collected from the 17 study populations and from two populations that were each adjacent to one of the study populations, but were not used in morphometrics (mean number of individuals per population = 6.0, range = 3–11; sequences were collected in two previous studies, Nosil et al. 2002, 2003).

Measures of Morphological Divergence

We derived multivariate indices of morphology using principal components (PC1; using correlation matrices) and canonical variate analyses (discriminant analyses). The latter method ordinates a priori groups (in this case, population of origin) so that it maximizes the between-group variation in relation to the within-group variation (unlike principal component analysis, which ordinates independently of trait contribution to between-group and within-group variation). Discriminant analysis is a powerful technique for the analysis of size-related characters as it overcomes the problem of information redundancy in the character set by taking into account the within-group covariation between characters. There was no evidence for host-associated divergence in the second or third axis (and these axes explained only up to 15% of the variance in morphology); thus, we focus our analysis of shape variation on the first canonical variate axes from these discriminant analyses (CV1 hereafter).

Second, we examined variation in single, size-corrected morphological traits using the residual values from a regression of each trait on PC1, as PC1 was a general index of body size (see Results). We report results using pooled among-group slopes for all cases except thorax width, where we report results using separate within-groups slopes (the relationship between trait size and PC1 tended to be homogeneous among study sites; $P > 0.10$ for the trait size $\times$ population interaction in all ANCOVA analyses except for thorax width, where $P < 0.05$).

We assessed multivariate population differentiation in morphology using nested ANOVA (PC1, CV1, single size-corrected traits) and nested MANOVA (all size-adjusted univariate traits). This method allowed us to estimate the amount of morphological variation attributable to variation between hosts, variation among populations within hosts, and variation within populations (error). In *T. cristinae*, the sexes are highly dimorphic in quantitative morphology and thus were treated separately in all cases.

Finally, we also tested whether interpopulation divergence in quantitative morphology is correlated with divergence in color-pattern morph frequency, a trait for which population differentiation has been previously shown to be under a balance between host-specific selection and gene flow (Sandoval 1994a,b). The 17 populations tested yielded 136 pairwise comparisons of divergence in color-pattern morph frequency and quantitative morphology. We tested for associations between populations distance matrices using the Mantel test, a nonparametric method that evaluates the strength of associations between matrices using randomization (Manly 1997). Significance levels were estimated using 10,000 randomizations. The Mantel program was designed by B. Manly and is commercially available through Western Ecosystems Technology (Cheyenne, WY).

Relative Population Sizes and the Geographic Potential for Gene Flow

Previous work in *T. cristinae* has shown that host-plant patch size and walking-stick population size are strongly and positively correlated ($r = 0.79$ and 0.73 for *Ceonothus* and *Adenostoma* patches, respectively; $n = 13$ patches of each host; data from Sandoval 1994a). In additions, levels of mitochondrial DNA gene flow into a study population, from its neighboring population, increase with increasing relative size of the host-plant patch used by the neighboring population ($r = 0.86$, 0.62, $P < 0.01$, 0.05, 0.01 for the effective number of migrants [*Nm*], migration rate estimated using effective population sizes [*m*], and the migration parameter M [$M = m$/mutation rate], respectively, $n = 8$; data from Nosil et al. 2003, obtained using the coalescent-based methods of Beerli and Felsenstein 2001). Consequently, the size of the host-plant patch occupied by a study population, relative to the size of the patch occupied by its neighboring population using the alternate host, reflects the geographic potential for gene flow into a study population. Patch sizes were estimated from aerial photographs (as in Sandoval 1994a; Nosil et al. 2003).

Predicting Mean Trait Values Using a Selection–Gene Flow Balance

We tested whether mean trait values for each single population reflect the effects of a balance between selection and gene flow using two, independent measures of gene flow: (1) geographic potential for gene flow, calculated as the relative sizes of the study populations and their neighboring population of the alternate host; and (2) coalescent-based estimates of migration rates between adjacent populations, calculated using mitochondrial DNA sequence variation.

Under the first method, when populations use *Ceonothus* as a host plant, the size of the neighboring population of *Adenostoma* serves as the index of the opportunity for gene flow to erode local adaptation to *Ceonothus*. Conversely, when populations use *Adenostoma* as a host plant, the size of the neighboring population of *Ceonothus* serves as an index of the opportunity for genes conferring adaptation to *Ceonothus* to be introduced into the population. Consequently, single *Adenostoma* populations were assigned values of [size of neighboring patch/(size of study patch + size of neighboring patch)]. Patches of *Ceonothus* represent a divergent selective regime and were assigned values of {1 − [size of neighboring patch/(size of study patch + size of neighboring patch)]}. Thus, for each study population the value assigned to it simply represents the proportion of the total area (study population area plus neighboring population area) occupied by *Ceonothus*.

Under the second, DNA-based methods, we used two independent approaches to derive indices of the balance between selection and gene flow. The first approach is based on estimating the proportion of individuals within a population that were derived from a *Ceonothus* population, using estimates of N_e and *Nm*. For populations using *Adenostoma* as a host plant, this value is represented by the proportion of individuals in the population that are estimated to be migrants from the neighboring population of the alternate host (m). Conversely, for populations using *Ceonothus* as a host plant, this value is represented by the proportion of individuals in the population that are not migrants ($1 - m$). We used our mitochondrial DNA sequence data to obtain estimates of m into each parapatric study population, from their neighboring population using the alternate host (with m assumed to be zero for allopatric populations). To begin, we estimated *Nm* using the methods of Beerli and Felsenstein (2001), which are tailored for estimating asymmetric migration rates between pairs of population and have less restrictive assumptions than F_{ST}-based methods (for discussion, see Whitlock and McCauley 1999). Second, we estimated m from *Nm* by calculating total population size (using previously published regression equations for patch size versus population size; Sandoval 1994a), and dividing this number by 0.5 to obtain female population size (mitochondrial DNA is maternally inherited). We note that although N is unlikely to be equal to N_e (Frankham 1995), our analyses depend only on variation in relative migration rates and are thus unaffected by N_e/N ratios (i.e., scaling N to N_e changes only the absolute estimates of m).

Under the second DNA-based approach, we used the migration parameter M ($M = m$/mutation rate), obtained from MIGRATE (Beerli and Felsenstein 2001), to derive an index of the balance between selection and gene flow. The relative combined area of a study population and its neighboring population occupied by *Ceonothus* was set to zero for allopatric *Adenostoma* populations and (arbitrarily) to 500 for allopatric *Ceonothus* populations. The value of M obtained from MIGRATE was assigned to parapatric *Adenostoma* populations, and parapatric *Ceonothus* populations were assigned a value of $500 - M$ (maximum M was 345). We note that because we conducted analyses of selection–gene flow balance using a nonparametric test (Spearman rank correlation; see below) changing the scaling factor (i.e., the value 500) does not affect our results. We stress that our analyses of the relationship between gene flow and neighboring population size depend only on relative (rather than absolute) migration rates.

Spearman rank correlations were used to test whether population mean values for PC1, CV1, and each size-adjusted single trait were correlated with our three indices of selection–gene flow balance. Due to the a priori expectation that gene flow would erode host-associated divergence, we report significance levels from one-tailed tests.

Rearing Environment and Morphological Variability

For a subset of the populations studied ($n = 6$), we raised some of the individuals on their native host and some on the alternative host (Table 1). We used two-way ANOVAs to test whether morphological variation among individuals from these populations was influenced by genotype (native population), rearing environment (host reared on), and a genotype-by-environment interaction. We attained congruent results when we used native host, rather than native population, as our genotype term.

Morphological Divergence and Reproductive Isolation

We assessed whether morphological divergence contributes directly to speciation by testing for a morphological basis to sexual isolation among populations of *T. cristinae*. Sexual

TABLE 2. Results of nested ANOVAs (PC1, CV1, single size-correct traits) and nested MANOVA (all size-adjusted traits) estimating the proportion of morphological variation attributable to variation between hosts and variation among populations within hosts. Also shown are mean (SD) trait values for populations using each host plant.

Trait	*F*-ratio hosts	Percent hosts	*F*-ratio populations	Percent populations	Mean (SD) *Ceonothus* populations	Mean (SD) *Adenostoma* populations
Males						
PC1	6.57*	14.5	8.35***	17.8	0.25 (0.99)	−0.27 (0.77)
CV1	5.92*	18.4	15.94***	28.4	0.47 (1.21)	−0.52 (1.20)
MANOVA (sc)						
ANOVA (sc)	11.46***	—	4.30***	—	—	—
Head width	0.99	2.5	3.39***	8.5	1.69 (0.05)	1.71 (0.05)
Femur length	24.27***	9.7	8.34***	18.8	2.97 (0.10)	2.90 (0.09)
Tarsal length	5.88*	2.4	3.04***	6.8	0.73 (0.05)	0.74 (0.04)
Abdominal length	4.26*	2.4	5.87***	14.8	8.80 (0.80)	9.04 (0.85)
Genitalia	1.18	0.0	2.38**	4.8	0.50 (0.04)	0.50 (0.04)
Subgenital plate	0.01	0.0	1.10	4.3	1.07 (0.07)	1.08 (0.06)
Thorax width	0.02	0.1	2.23**	3.8	2.85 (0.13)	2.85 (0.12)
Females						
PC1	11.08**	23.3	7.64***	14.8	0.25 (0.93)	−0.29 (1.00)
CV1	8.62**	25.0	13.99***	23.9	0.44 (1.22)	−0.51 (1.12)
MANOVA (sc)						
ANOVA (sc)	11.66***	—	4.05***	—	—	—
Head width	46.94***	23.5	7.89***	15.2	3.42 (0.80)	3.91 (0.88)
Femur length	11.05**	3.3	11.39***	26.3	3.81 (0.12)	3.75 (0.11)
Tarsal length	0.34	0.0	1.18	0.6	0.95 (0.05)	0.95 (0.05)
Abdominal length	4.84*	1.6	4.64***	11.6	13.70 (1.51)	14.15 (1.42)
Genitalia	3.55	2.3	0.96	0.0	2.89 (0.13)	2.91 (0.13)
Subgenital plate	0.32	1.2	2.45**	5.0	2.64 (0.21)	2.68 (0.19)
Thorax width	10.78**	7.2	2.54**	4.9	4.97 (0.22)	4.89 (0.25)

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

isolation was estimated in a previous study, using no-choice mating trials. One male and one female were placed in a 10-cm petri dish and at the end of 1 h we scored whether the male and female were paired (male on female without genital contact) or not, and copulating or not (for details, see Nosil et al. 2002).

In the current study, we restrict our analyses to between-population mating trials, as we are interested in the potential influence of morphological divergence between populations on the probability of interbreeding between populations. First, we used logistic regression to test if the difference in trait values between a male-female pair influenced the probability of copulation (significance tested using likelihood ratio tests, all df = 1). Adding population-pair as a factor (see below) in the logistic regression yielded no significant main effects or interaction terms and thus is excluded from the analyses presented.

Second, we tested for assortative mating by morphology in the between-population mating trials that resulted in copulation. Among copulating pairs, ANCOVAs were used to determine whether male trait values were correlated with female trait values and to test whether such a relationship differed among the 28 different pairs of populations examined (i.e., test for homogeneity of slopes); in Nosil et al. (2002), sexual isolation was estimated for all pairwise comparisons between eight of the populations in the current study, yielding a total of 28 pairwise comparisons between populations that pertain to the current study. We report results from analyses using both single traits and our multivariate indices of size and shape. All statistical analyses were conducted using SPSS (ver. 10.1; Chicago, IL).

RESULTS

Morphological Divergence between Hosts and among Populations

Populations of *T. cristinae* from *Adenostoma* differed significantly in multivariate morphology from those on *Ceonothus* (Table 2). Although populations using the same host tended to be morphologically similar, a significant proportion of the morphological variation was also partitioned among populations using the same host (e.g., significant variation between hosts and among populations within hosts; Table 2). For example, for both PC1 and CV1 comparable proportions of morphological variation were partitioned between hosts and among populations within hosts (Table 2; see below for description of these indices of morphological variation). Graphical analyses confirmed that the study populations exhibit both host-specific and population-specific variation in morphology: while populations using the same host tend to cluster together in morphospace, two *Adenostoma* populations were situated near the bulk of the *Ceonothus* populations and three *Ceonothus* populations were situated near the bulk of the *Adenostoma* populations (Fig. 1). Analysis of single, size-adjusted trait values were congruent with multivariate results; for most traits, we observed both host-specific and population-specific morphological variation (Table 2).

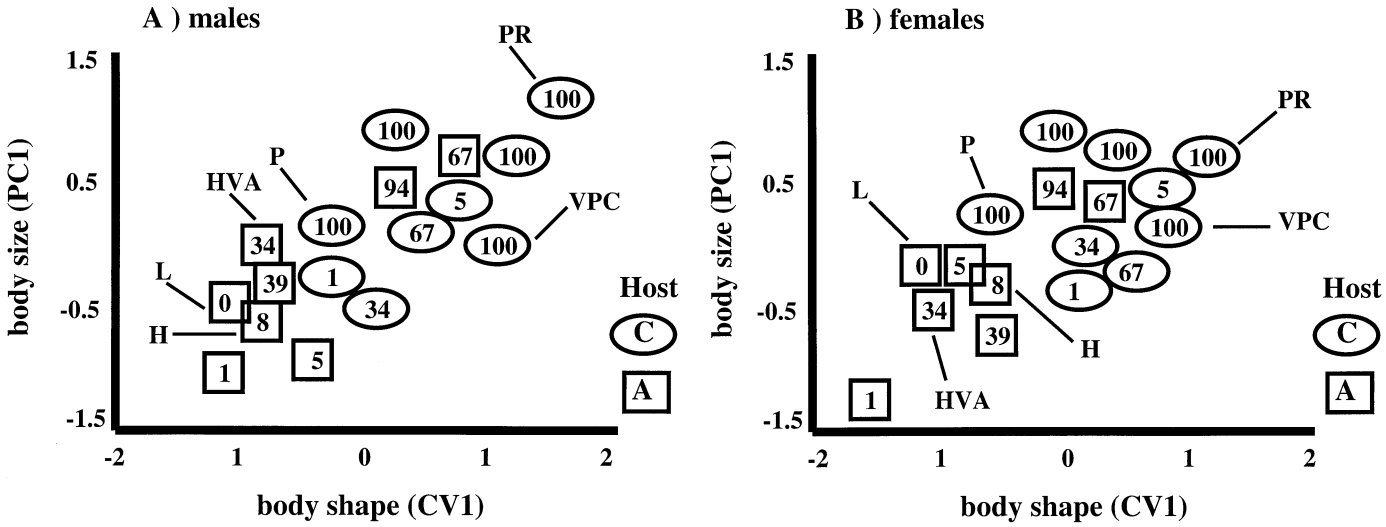

FIG. 1. Interpopulation divergence in morphology for 17 host-associated populations of *Timema cristinae*. Values shown are population means for PC1 (an index of overall body size) and group centroids for CV1 (an index of shape, see Table 3 for trait loadings). Marker depicts which host a population utilizes: ovals, *Ceonothus spinosus*; squares, *Adenostoma fasciculatum*. Some populations have a neighboring population of the alternative host (parapatric sites), while others do not (allopatric sites). The proportion of the combined area of a study population and its neighbor that is occupied by *Ceonothus* is shown within each maker (e.g., 0 and 100 for allopatric *Adenostoma* and *Ceonothus* populations, respectively). Population codes are shown for the six populations used in the common-garden experiment. (A) Males; (B) females.

Indices of Morphological Divergence

In a principal components analysis, all the measured traits exhibited high and positive loadings for PC1, indicating that PC1 largely reflects variation in general body size (Table 3).

In a discriminant analysis, the first canonical variate axis (CV1) explained 58% of the variance in male morphology. CV1 exhibited high positive loadings for femur and tarsal lengths but a strong negative loading for head width, indicating that walking-sticks with high scores for CV1 are characterized by narrow heads and long legs (Table 3). For females, the first canonical variate axis in the discriminant analysis explained 55% of the variance. As in males, CV1 exhibited a high positive loading for femur length but a high negative loading for head width, indicating that the same traits contribute to between-population shape variation in both sexes (Table 3; correlation between group centroids in females and group centroids in males, $r = 0.93$, $P < 0.001$).

TABLE 3. Principal component scores for the first principal component axis (PC1) and standardized canonical discriminant function coefficients for the first canonical variate axis (CV1; from principal components and discriminant analyses, respectively). In both sexes, high scores for CV1 characterize walking-sticks with narrow heads and long legs.

Trait	Males PC1	Females PC1	Males CV1	Females CV1
Head width	0.79	0.86	−0.47	−0.51
Femur length	0.82	0.88	1.17	1.39
Tarsal length	0.65	0.78	0.20	0.06
Abdominal length	0.45	0.48	−0.03	−0.37
Genitalia[1]	0.36	0.78	−0.16	−0.13
Subgenital plate	0.63	0.65	−0.11	−0.26
Thorax width	0.72	0.76	0.03	0.12

[1] Genital length for females, genital width for males.

We detected host-specific morphological divergence in body size (PC1) and shape (CV1) in both males and females. Specifically, PC1 scores were higher for individuals from populations using *Ceonothus* than for individuals from populations using *Adenostoma* (Table 2). Likewise, CV1 scores were higher for individuals from populations using *Ceonothus* than for individuals from population using *Adenostoma*. Thus, individuals from populations using *Ceonothus* tended to be larger in overall body size and have relatively small heads and long legs.

Analyses of single, size-adjusted traits were congruent with multivariate analyses; individuals from populations using *Ceonothus* tended to exhibit smaller, size-adjusted head widths and larger, size-adjusted femur lengths (Table 2).

Predicting Mean Trait Values Using a Selection–Gene Flow Balance

Mean trait values for each population could be predicted by host-plant used and the opportunity for homogenizing gene flow. Thus, population means for PC1 (body size) and CV1 (body shape) were significantly correlated with both the geographic (% total area occupied by *Ceonothus*) and DNA-based indices of the balance between selection and gene flow (males and females, all $P < 0.05$, Table 4). Moreover, population means for the two traits that contribute most to CV1 were often correlated with our indices of the balance between selection and gene flow (size-adjusted head width and size-adjusted femur length, Table 4; all other size-adjusted traits $P > 0.05$ in all cases). Thus, homogenizing gene flow from neighboring populations of the alternate host accounts for the morphology of populations that exhibit size and shape variation indicative of populations using the alternate host (Fig. 1).

TABLE 4. Population means for PC1 and CV1 are correlated with geographic and molecular-genetic indices of the opportunity for divergence, under a balance between natural selection and gene flow (shown are *r*-values from Spearman rank correlation analyses). The opportunity for divergence was calculated from the size of adjacent populations using the alternate host (geographic method) and DNA estimates of migration rates between adjacent patches (DNA-based methods; see Materials and Methods for derivation of these indices). Also shown are results for the two size-adjusted, univariate traits where a significant relationship was detected between morphological divergence and indices of the balance between selection and gene flow.

	PC1	CV1	HW (sc)	FL (sc)
Males, selection–gene flow balance				
Geographic method	0.74***	0.65**	−0.43**	0.39
DNA-based method 1 (N_e)	0.66**	0.69**	−0.41**	0.54*
DNA-based method 2 (M)	0.64**	0.72**	−0.42**	0.58**
Females, selection–gene flow balance				
Geographic method	0.69**	0.52*	−0.72**	0.22
DNA-based method 1 (N_e)	0.79***	0.66**	−0.81***	0.25
DNA-based method 2 (M)	0.78***	0.62**	−0.80***	0.23

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

Because mean PC1 and CV1 values for each population were correlated with one another ($r = 0.79$, 0.71 for males and females respectively, both $P < 0.01$), we assessed the effects of selection–gene flow balance on each morphological variable independently in multivariate analyses (separate analyses for each sex; PC1 and CV1 treated as independent variables). Multiple regression models including both PC1 and CV1 were significant overall for both the geographic and the DNA-based indices of selection–gene flow balance (all $r > 0.60$, all $P < 0.05$). We were able to statistically distinguish the independent contributions of PC1 and CV1 to the overall model only for the geographic selection–gene flow index in males (PC1, $B = 41.63$, SE $B = 19.79$, $P < 0.05$; CV1, $B = 8.97$, SE $B = 14.66$, $P = 0.55$) and for the DNA-based selection–gene flow index in females (PC1, $B = 5.11$, SE $B = 17.09$, $P = 0.77$; CV1, $B = 38.21$, SE $B = 17.33$, $P < 0.05$; individual contribution of PC1 and CV1 nonsignificant in the other two regression analyses, all partial $r < 0.10$, all $P > 0.10$). Thus, both size and shape variation among populations appears to be influenced by a balance between selection and gene flow.

Finally, population differentiation in color-pattern morph frequency was significantly correlated with population differentiation in PC1 (Mantel test, $r = 0.37$, 0.29 for males and females respectively, both $P < 0.001$) and CV1 ($r = 0.52$, 0.38 for males and females respectively, both $P < 0.001$). We note that mean PC1 and CV1 scores do not differ between color-pattern morphs within populations for males (all $P > 0.10$, nested ANOVA) but do differ between color-pattern morphs within populations for females (mean PC1 = 0.31, −0.33, mean CV1 = 0.36, −0.40 for unstriped and striped morphs, respectively; $F_{15.425} = 1.98$, 172 for PC1 and CV1, respectively; both $P < 0.05$; nested ANOVA). Thus, quantitative morphology is likely to have diverged among populations via direct selection on size and shape, selection on a correlated trait (i.e., color pattern), or some combination of these processes.

Rearing Environment and Morphological Variability

The results of the common-garden experiment revealed that size and shape variation apparently have a partial genetic basis (highly significant genotype term for all variables; Table 5; Fig. 2). Environmental effects, when detected, were interactive with genotype or much weaker than the effects of genotype. For example, for PC1 in both males and females, the effects of genotype (native population) were interactive with the effects of environment (host reared on). Individuals from *Ceonothus* populations tended to grow larger when reared on their native host than when reared on the alternate host, whereas the morphology of individuals from *Adenostoma* was unaffected by rearing environment (Fig. 2).

Morphological Divergence and Reproductive Isolation

There was no evidence that population divergence in morphology contributed to the evolution of reproductive isolation. When the sexes were from different populations, the probability of copulation was not influenced by the difference between them in body size (likelihood-ratio [LR] tests from logistic regression analyses; same-host pairs, LR = 1.69, P

TABLE 5. Results of ANOVAs of common-garden rearing experiment testing for the effects of genotype (population of origin), environment (host reared on), and the genotype-by-environment interaction term (population × host reared on) on morphological variation in *Timema cristinae*. Figure 2 depicts mean trait values for each multivariate index of morphology for the six study populations, where some individuals were reared to maturity on their native host and some individuals were reared on the alternate host.

	Males		Females	
Population	PC1	CV1	PC1	CV1
Population of origin	10.80****	33.77***	9.26***	32.39***
Host reared on	3.68	0.30	13.23***	11.44**
Population × host reared on	8.03****	0.99	7.61***	1.30

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

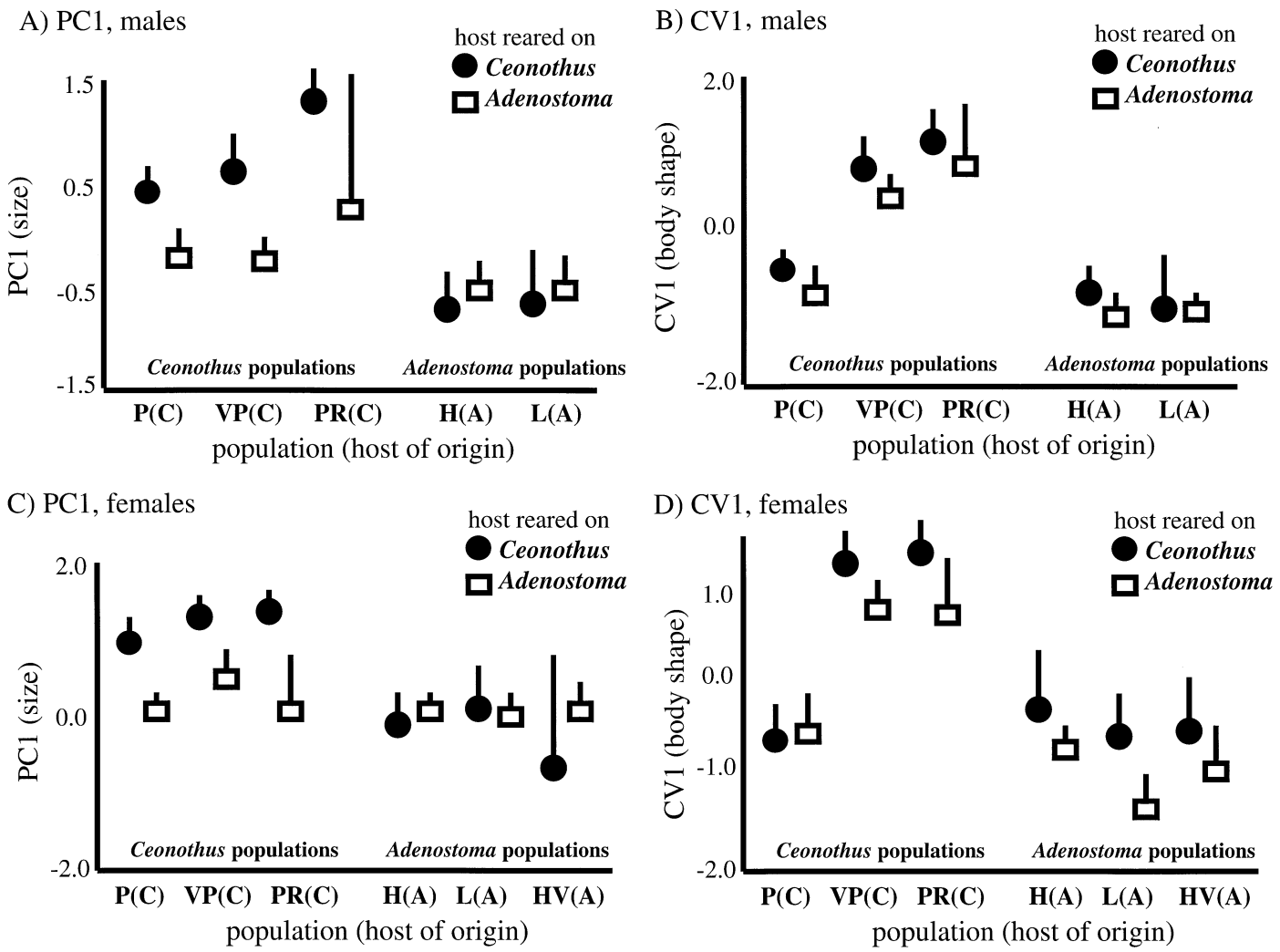

FIG. 2. Mean body size (PC1) and body shape (CV1) for walking-sticks collected from *Ceonothus* and *Adenostoma* and reared in the laboratory on either their native or the alternate host. Mean trait values (± 95% CI) are shown for each population, and statistical results are presented in Table 5. Males from HV(A) are not shown, as only two individuals were reared on *Ceonothus* successfully.

= 0.19, n = 416; different-host pairs, LR = 0.01, P = 0.99, n = 480) or body shape (same-host pairs, LR = 1.90, P = 0.17; different-host pairs, LR = 0.06, P = 0.81; $P > 0.05$ for all single traits as well). Moreover, in the between-population mating trials that did result in copulation, there was no evidence for assortative mating by morphology; for all the variables examined, the relationship between male trait values and female trait values did not differ among the 28 pairs of populations tested (all $P > 0.05$, ANCOVA test for homogeneity of slopes) and it was not significant overall in any case (all $r < 0.10$, all $P > 0.05$).

DISCUSSION

We detected host-specific morphological divergence in *T. cristinae*: on average, individuals from populations using *Adenostoma* as a host plant exhibited smaller overall body size and shorter legs and wider heads than individuals from populations using *Ceonothus* as a host. However, there was also significant variation in morphology among populations within host-plant species, which was associated with variability in levels of gene flow between populations using alternate hosts. Thus, the degree of morphological differentiation observed among populations of *T. cristinae* reflects a balance between host-specific selection and homogenizing gene flow.

Natural Selection and Host-Associated Morphological Divergence

Multiple lines of evidence implicate natural selection as the cause of morphological differentiation in *T. cristinae*. First, morphometric differences between allopatric populations using different hosts were always in the same direction; genetic drift is highly unlikely to cause different populations in similar environments (i.e., hosts) to converge on similar morphologies. A role for selection is strengthened if similar traits have evolved independently, via parallel evolution, in multiple populations that inhabit similar environments (Schluter and Nagel 1995). Phylogenetic analysis of mitochondrial and nuclear DNA (Nosil et al. 2002), coupled with population-genetic and nested- cladistic analyses (P. Nosil and B. J. Crespi, unpubl. data), indicate that divergence in

host-plant use among these *T. cristinae* populations has occurred multiple times. Thus, morphological divergence has occurred repeatedly and in parallel with divergence in host-plant use, strongly implicating selection as the cause of evolution.

Second, phylogeographic and molecular-genetic data also indicate that differentiation via genetic drift is unlikely to have caused the greater morphological divergence observed between pairs of populations using different versus the same host plant. Pairs of populations using different host plants are not more differentiated in mitochondrial DNA or at a nuclear locus (ITS-2) than are pairs of populations using the same host plant (Nosil et al. 2002). Moreover, levels of gene flow between adjacent pairs of *T. cristinae* populations are generally too high to allow differentiation via genetic drift (e.g., $Nm > 1$; Nosil et al. 2003). For example, previous work on these *T. cristinae* populations has shown that adjacent pairs of populations using different host plants are weakly or not differentiated at mitochondrial DNA, while geographically separated populations are strongly differentiated (mean $F_{ST} = 0.07$, 0.31, respectively; Nosil et al. 2003), indicative of substantial gene flow between neighboring populations.

Finally, consideration of functional design suggests that host-specific differences in size and shape represent adaptations to divergent predation regimes, with small body size being important for crypsis when resting against the thin, needlelike leaves of *Adenostoma* but not against the broad leaves of *Ceonothus*. The importance of small size for crypsis on *Adenostoma* is supported by divergence in color pattern between populations of *T. cristinae* using different hosts. Thus, populations using *Adenostoma* exhibit a much higher frequency of the striped color-pattern morph than do *Ceonothus* populations, and the striped morph is much more cryptic on *Adenostoma* than is the unstriped morph (Sandoval 1994a,b). The thin, white, longitudinal stripe along the dorsal surface of this morph apparently functions as disruptive coloration, breaking up the insect's body into smaller segments and improving crypsis against the thin leaves of *Adenostoma*. Such disruptive coloration is expected and common among other cryptic animals that live in heterogeneous environments (Endler 1990; Merilaita et al. 1999).

Adenostoma and *Ceonothus* are also very structurally different, with *Ceonothus* plants being larger, woodier, and more treelike than the small, bushlike *Adenostoma* plants. Host-specific morphological differences could also be related to differences in the types of morphology that facilitate efficient movement and maneuvring on structurally different plants (e.g., Moran 1986; Bernays 1991). Under either of the above scenarios, fecundity selection for larger size in females (Leather 1988) could be offset by host-specific selection for smaller size on *Adenostoma*, exerted either by visual predators or by host-plant surfaces. Notably, host-specific natural selection, rather than genetic drift, has caused the evolution of reproductive isolation (Nosil et al. 2002) and possibly physiological divergence, as shown by the genotype-by-environment interactions detected in this study.

The results of our common-garden experiment suggest that the traits examined have a genetic basis. Because each test animal was born in the wild and spent a brief period on its native host prior to capture in the first instar, some of the morphological variation observed might be attributable to maternal effects (Mousseau and Dingle 1991) or environmental induction (e.g., Gillham and Claridge 1994). However, size and shape are likely to be under at least partial genetic control because: (1) the time spent on the native host in the field is negligible relative to the time spent being reared in the laboratory;(2) the magnitude of the genotype effects was large and highly significant (this result is independent of environmental, but not maternal, effects); (3) environmental effects on morphology were either nonsignificant, interactive with genotype, or much weaker than the effects of genotype; (4) the results of common-garden experiment were highly consistent across the six populations tested; (5) previous studies have consistently revealed a genetic basis to morphological variation in other insects (e.g., Carroll and Boyd 1992; Arnqvist and Thornhill 1998); and (6) if size and shape in *T. cristinae* did not have a genetic basis, it is exceedingly unlikely that the pattern of variation in natural populations would conform to that expected under a balance between selection and gene flow. Due to relatively low absolute migration rates (Sandoval 1993; Nosil et al. 2003), most individual *T. cristinae* spend their entire development on the same host-plant species; environmental or maternal effects on morphology cannot account for why (as a result of gene flow) some populations using *Adenostoma* exhibit the morphology typical of *Ceonothus* populations and vice versa.

Collectively, our results implicate host-specific natural selection as the cause of morphological divergence in *T. cristinae*. Measurements of selection will allow a direct test of this hypothesis (Lande and Arnold 1983; Endler 1986), estimation of the degree to which gene flow prevents populations from attaining optimal trait values (cf. Hendry et al. 2001), and an assessment of whether host-associated divergence in quantitative morphology represents a correlated response to divergent selection on color pattern within populations, given that female morphology differed between color-pattern morphs within populations. Studies of phytophagous insects have traditionally focused on detecting evidence for physiological adaptations to the use of different host plants (Rausher 1982; Via 1989; Sheck and Gould 1993; Craig et al. 1997). Our results suggest that host-specific selection on insect morphology may also be common (see also Moran 1986; Bernays 1991; Carroll and Boyd 1992).

Parallels with Previous Studies of Selection–Gene Flow Balance

Previous work on a wide range of taxa has demonstrated inverse associations between trait divergence and levels of gene flow (e.g., fish: Lu and Bernatchez 1999; Hendry et al. 2002; Saint-Laurent et al. 2003; amphibians: Storfer and Sih 1998; Storfer et al. 1999; birds: Dhondt et al. 1990; Smith et al. 1997; reptiles: King and Lawson 1995; insects: Sandoval 1994a; Ross and Keller 1995; arachnids: Riechert 1993; Riechert and Hall 2000; Riechert et al. 2001). Moreover, such associations have been documented for morphology (e.g., Sandoval 1994a; Lu and Bernatchez 1999; Hendry et al. 2002; Saint-Laurent et al. 2003), behavior (e.g., Riechert 1993; Storfer and Sih 1998; Storfer et al. 1999; Riechert et al. 2001), and life-history traits (Dhondt et al. 1990), and the traits

examined span a wide range of functions (e.g., antipredator defense: Sandoval 1994; Storfer and Sih 1998; Storfer et al. 1999; foraging ability: Hendry et al. 2002; flight: Smith et al. 1997; agonistic behavior: Riechert 1993; Riechert and Hall 2000; Riechert et al. 2001). Inverse associations between gene flow and trait divergence thus appear to be common and widespread in nature, suggesting that trait divergence often reflects a balance between selection and gene flow.

Our study expands on previous studies of selection–gene flow balance by testing whether the traits examined also influence mate choice, making it possible to infer the degree to which trait divergence is a cause versus a consequence of reduction in gene flow. We detected no evidence that body size or shape influences the probability of copulation in between-population mating trials, indicating that morphological divergence among populations does not reduce gene flow via the evolution of sexual isolation. Thus, morphological divergence is more likely to be a consequence than a cause of reductions in gene flow. Given the ability of our mating data to elucidate the causes of variation in levels of reproductive isolation (see Nosil et al. 2002, 2003) and given the large size of our samples, this result is unlikely to stem from a lack of statistical power. This same causal association exists for color-pattern morph frequency in *T. cristinae*, where the frequency of the more cryptic morph within a patch is inversely related to the potential for gene flow from adjacent patches of the alternate host (Sandoval 1994a) and color pattern is also not used in between-population mate choice (Nosil et al. 2002). Although morphological divergence does not reduce gene flow via premating isolation, the effects of morphological divergence on ecologically dependent postmating isolation (i.e., reduced hybrid fitness; Rundle and Whitlock 2001; Rundle 2002) are unknown and offer promising avenues of further research.

Finally, we note that a large proportion of geographic variation in morphology remained unexplained by our indices of the balance between selection and gene flow. Several processes could account for this unexplained variation, including variation in the strength of selection among populations of the same host, inaccuracy in our estimates of gene flow, and rare episodic instances of gene flow into the currently allopatric populations.

Conclusions

Our results have broad implications for studies of natural selection and the causes of speciation. Although assortative mating by size is common among insects (Crespi 1989) and despite high levels of reproductive isolation among populations of *T. cristinae* (Nosil et al. 2002, 2003), we did not detect size- or shape-assortative mating within this species. Moreover, previous studies have shown that the traits used in within-populations mate choice do not always contribute to between-population mating discrimination or to species recognition (e.g., Claridge and Morgan 1993; Boake et al. 1997; Nosil et al. 2002). Collectively, these findings indicate that data on between-population mating preferences are required to determine if and how interpopulation trait differentiation is causally related to the evolution of premating isolation.

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