

$\sum(\Delta R/\bar{R})^2$, which the model predicts will be greater for less abundant species, and allows for differences in lifetimes and sample sizes. The second term corrects for merely statistical fluctuation, assuming Poisson statistics. For each contrast, the estimator was summed over age classes for each species.

Bursera instabilis is more closely related to *Bursera arborea* than either is to *Bursera excelsa*³⁰. Therefore a higher level contrast was formed to compare *B. excelsa* to a single value of the two other congeners (*Bursera* 2). A weighted pooling of the data for *B. instabilis* and *B. arborea* was based on the abundance of the former relative to the latter, drawing on the ecological assumption that the more distantly related *B. excelsa* would interact with the two other, closely related species as a single functional entity.

Samples represented a larger proportion of the populations of the rarer than the more common species. We therefore compared our observed results with a null model in which a subsample of a size corresponding to the sampling intensity of the less heavily sampled species was randomly selected without replacement from the list of age values for the more heavily sampled species. Deviation of this subsample from a smooth curve was determined as for the observed populations, repeated 10⁴ times. In all five instances, the null model showed that for the less abundant, more intensively sampled species, a less intensive sampling regime produced a value that did not differ significantly from observed (analysis of variance; $P \geq 0.80$ in all cases).

We compared synchrony within contrast pairs in fluctuation patterns in a sign test, asking whether age classes of comparable taxa deviated in concert from the expected distribution for the taxon significantly more often than expected by chance.

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Host-plant adaptation drives the parallel evolution of reproductive isolation

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Parallel evolution of similar traits in independent populations that inhabit ecologically similar environments strongly implicates natural selection as the cause of evolution¹. Parallel speciation is a special form of parallel evolution where traits that determine reproductive isolation evolve repeatedly, in closely related populations, as by-products of adaptation to ecological conditions^{1,2}. The outcome of such parallel evolution is that ecologically divergent pairs of populations exhibit greater levels of reproductive isolation than ecologically similar pairs of populations of a similar or younger age^{2–4}. The parallel evolution of reproductive isolation provides strong evidence for natural selection in the process of speciation¹, but only one conclusive example from nature is known². Populations of the walking-stick insect *Timema cristinae* that use different host-plant species have diverged in body size and shape, host preference, behaviour and the relative frequency of two highly cryptic colour-pattern morphs^{5,6}. Here we report that divergent selection for host adaptation, and not genetic drift, has promoted the parallel evolution of sexual isolation in this species. Our findings represent a clear demonstration that host-plant adaptation can play a crucial and repeatable role in the early stages of speciation.

Timema walking-stick insects are wingless, phytophagous insects that inhabit the chaparral of California, other areas of the western United States, and northern Mexico. Species are monomorphic or polymorphic for colour, presence of a dorsal stripe, or both, and these patterns match those of their host plants^{5–7}. Phylogenetic studies suggest that speciation in this genus has involved specialization on different host plants⁷. *Timema cristinae* exhibits two genetically determined⁸ colour-pattern morphs, with an unstriped morph more common on *Ceanothus spinosus* and a striped morph more common on *Adenostoma fasciculatum* (mean frequency of unstriped morph on *Ceanothus* is 81%, range 33–100%; mean frequency of unstriped morph on *Adenostoma* is 28%, range 0–100%). Predation on *T. cristinae* by birds and lizards is strong and each morph is most cryptic on the host plant on which it is more common^{5,6}. Thus populations of *T. cristinae* using different host-plant species are exposed to intense divergent selection for crypsis. Patches of these two host plant species are distributed in parapatric mosaics, and local morph frequencies are determined by a gene flow–selection balance between host-plant patches exhibiting the different selective regimes⁶.

Local adaptation to host-plant species in *T. cristinae* is also

indicated by a number of other phenotypic traits that are more divergent between pairs of populations using different host plants than between pairs using the same host. These traits include body size (first principal component, PC1: males, Mantel test $t = -1.86$, $P < 0.05$; females, Mantel test $t = -1.49$, $P < 0.05$), body shape (CVA1: males, Mantel test $t = -1.51$, $P < 0.05$; CVA2: females, Mantel test $t = -1.69$, $P < 0.05$; first and second canonical variate axes from population-level discriminant analyses), host preference⁸ and cryptic resting behaviour (per cent resting where visible from above, the side and below, respectively; from *Ceanothus*: 36%, 33%, 31%; from *Adenostoma*: 16%, 70%, 15%; χ^2 , 2 degrees of freedom (d.f.) = 62.34, $P < 0.001$, $n = 537$). Such host-specific differentiation suggests that host-plant adaptation involves divergence in a suite of complex morphological and behavioural traits. However, physiological trade-offs in host-plant use have not been detected, with females of both morphs exhibiting higher fecundity on *Ceanothus*⁸.

Phylogenetic analyses indicate that different populations using the same host plant do not form monophyletic groups (Shimodaira–Hasegawa tests⁹: on *Adenostoma* the difference in ln likelihood = 77.37 for mitochondrial DNA (mtDNA) (cytochrome oxidase I gene (COI)), 22.57 for nuclear DNA (internal transcribed spacer 2 (ITS-2)), both $P < 0.001$; on *Ceanothus* the difference in ln likelihood = 75.49, $P < 0.001$ for mtDNA, 6.45, $P = 0.08$ for nuclear DNA; Templeton tests¹⁰: $P < 0.05$ for all four analyses). Thus the use of different host-plant species in *T. cristinae* has evolved many times, allowing a replicated test of whether sexual isolation has evolved in parallel with ecological divergence in host-plant use.

No-choice mating trials were conducted between all possible combinations of eight allopatric (geographically separated) populations, yielding a total of 28 pairwise comparisons of sexual isolation. The results revealed strong evidence of host-associated sexual isolation: across all populations, walking-stick insects were more likely to copulate if paired with an opposite-sex member from the same host-plant species than if paired with an opposite-sex member from the different host-plant species (Fig. 1; male host by female host interaction, likelihood ratio test (LR) = 23.98, 1 d.f., $P < 0.001$, logistic regression, $n = 1,024$ mating trials). Consistent with the main prediction of the parallel speciation hypothesis, the magnitude of sexual isolation detected between pairs of populations

using different host plants (I_{PSI} , an overall sexual isolation index = 0.24, s.d. = 0.14, $n = 15$) was significantly greater than the magnitude of isolation detected between pairs of populations using the same host plant ($I_{PSI} = 0.08$, s.d. = 0.10, $n = 13$) (Mantel test $t = 2.24$, $P < 0.05$; Table 1).

The degree of sexual isolation observed depended strongly on which pairs of populations were compared (LR = 13.61, 4 d.f., $P < 0.01$, logistic regression). We tested three hypotheses concerning the causes of this variation. First, interpopulation differences in colour-pattern morph frequency (per cent difference in frequency of unstriped morph) were positively correlated with the magnitudes of sexual isolation observed between pairs of populations ($r = 0.38$, $P < 0.05$, Mantel test). This result strongly suggests that the net strength of divergent selection for host adaptation to which populations are exposed directly influences the actual magnitude of sexual isolation that evolves. Stronger divergent selection will translate into local adaptation and the evolution of higher levels of sexual isolation. Conversely, gene flow between patches will tend to erode local adaptation⁶ and progress towards speciation.

We found that the geographic distance between populations was positively correlated with the degree of sexual isolation detected ($r = 0.37$, $P < 0.05$, Mantel test), which suggests that geographic isolation facilitates the evolution of sexual isolation. We also found that the degree of sexual isolation observed between populations was not correlated with genetic distances between pairs of populations (mtDNA: $r = 0.13$, $P = 0.29$; nuclear DNA: $r = 0.29$, $P = 0.10$, Mantel tests), and that pairs of populations using different host plants were not more genetically divergent from one another (mean genetic distance, mtDNA: 2.80%, s.d. = 0.81; nuclear DNA: 0.88%, s.d. = 0.83) than pairs of populations using the same host plant (mean genetic distance, mtDNA: 3.07%, s.d. = 0.79; nuclear DNA: 1.10%, s.d. = 0.83; Mantel test $t = 0.47$ and 0.76, respectively, both $P > 0.15$). Given that a molecular clock could not be rejected for the best maximum likelihood tree inferred from the mtDNA data set, variability in the length of time available for genetic drift to differentiate same-host versus different-host populations of *T. cristinae* is therefore unlikely to account for the increased sexual isolation detected between ecologically divergent populations.

Our work indicates that divergent natural selection and sub-

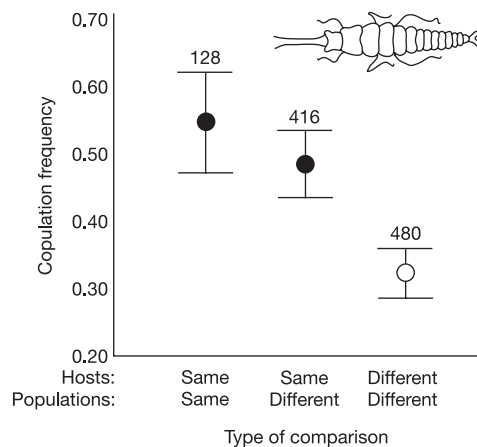


Figure 1 Copulation frequencies were higher for ecologically similar pairs of *T. cristinae* walking-stick insects than for ecologically divergent pairs, where divergence refers to differences in host-plant use ($P < 0.001$, logistic regression). Numbers of mating trials for each pair type are shown above each bar. In between-population mating trials, morph pair-type did not influence the probability of copulation (copulation frequencies when males and females were the same morph and different morphs: different-host crosses,

35%, $n = 219$ and 34%, $n = 261$, respectively, $-2[\log]$ likelihood = 0.18, 1 d.f., $P = 0.67$; same-host crosses, 44%, $n = 218$ and 51%, $n = 198$, respectively, $-2[\log]$ likelihood = 2.01, 1 d.f., $P = 0.17$). This result suggests that colour pattern is not used during mate choice in between-population tests and that host-plant adaptation, rather than selection for crypsis, has directly promoted the evolution of sexual isolation between populations.

Table 1 Levels of sexual isolation between populations of *T. cristinae*

Host comparisons	I_{PSI} (s.d.)	Likelihood ratio test
Different host		
VPC × HV	0.52 (0.19)*	9.99†
VPC × OUTA	0.01 (0.21)	0.01
VPC × L	0.20 (0.21)	2.27
VPC × H	0.11 (0.24)	0.17
VPC × VPA	0.20 (0.21)	1.60
PR × VPA	0.12 (0.20)	1.01
PR × L	0.39 (0.18)*	7.85†
PR × H	0.11 (0.21)	0.31
PR × OUTA	0.36 (0.20)	4.43*
PR × HV	0.20 (0.97)	3.23
P × H	0.19 (0.20)	2.27
P × OUTA	0.16 (0.19)	1.61
P × L	0.39 (0.17)*	9.22†
P × HV	0.33 (0.16)*	7.85†
P × VPA	0.41 (0.18)*	8.09†
Same host		
PR × P	0.08 (0.8)	0.60
PR × VPC	0.00 (0.20)	0.06
P × VPC	0.01 (0.20)	0.07
VPA × OUTA	0.07 (0.19)	0.25
H × VPA	-0.02 (0.20)	0.00
H × HV	0.06 (0.19)	0.64
H × OUTA	-0.11 (0.20)	0.57
HV × VPA	0.28 (0.19)	7.00†
HV × OUTA	0.11 (0.18)	0.48
L × VPA	0.13 (0.17)	1.01
L × H	0.08 (0.19)	0.58
L × OUTA	0.10 (0.20)	1.05
L × HV	0.20 (0.17)	2.01

I_{PSI} is an overall sexual isolation index for each pairwise comparison (range -1 to +1; null = 0, +1 = complete sexual isolation). Likelihood ratio values are from likelihood-ratio tests of the male population × female population interaction term in a logistic regression model, where a significant interaction term indicates sexual isolation. Using both analyses, stronger sexual isolation was detected for different-host comparisons than for same host-comparisons (see also Fig. 1). $n = 64$ mating trials for each pairwise comparison. Population using *Adenostoma* are italicized; eight populations were used in total. Population codes and locations available from P.N.

* $P < 0.05$.
† $P < 0.01$.

sequent host adaptation, rather than genetic drift, has promoted the parallel evolution of sexual isolation. Visual predation has a crucial role in promoting sexual isolation among populations of *T. cristinae* by limiting gene flow between populations using different hosts. Three processes contribute to this reduction of gene flow. First, between-host migrants will be locally less cryptic and will exhibit reduced survivorship owing to predation^{5,6}. Second, mating success of between-host migrants will be reduced because *T. cristinae* mates assortatively by colour morph within populations and the less cryptic morph is rare (interpopulation mating experiment: $I_{PSI} = 0.46$, $n = 7$ pairs; additional laboratory matings: $I_{PSI} = 0.65$, $n = 128$ trials; copulating pairs collected in the wild: in *Ceanothus* populations, $I_{PSI} = 0.57$, $n = 88$, in *Adenostoma* populations, $I_{PSI} = 0.43$, $n = 64$; I_{PSI} measures sexual isolation between morphs within populations, all $P < 0.05$). Finally, even if locally less cryptic migrants secure mates, they will produce a higher proportion of locally less cryptic offspring because colour morph is genetically determined⁸. Given that host adaptation promotes sexual isolation and that gene flow erodes local adaptation⁶, differential predation accelerates the evolution of reproductive isolation by reducing the homogenizing effects of gene flow and facilitating adaptive differentiation through natural selection. □

Methods

Study species and location

Timema cristinae were collected from eight study sites in the Santa Ynez Mountains of California between February and April 2001. Individuals from different populations and the sexes were kept separate. Animals were fed foliage of the host on which they were collected and all the test animals used in mating experiments were juveniles that were reared to maturity in the laboratory.

Differentiation in morphology and cryptic resting behaviour

We conducted seven linear measurements on 550 walking-stick insects ($n = 259$ males,

291 females). Each trait was measured twice and measurement error was low for all traits (all repeatabilities greater than 0.90, $P < 0.001$, analysis of variance (ANOVA)). Mantel tests¹¹ were used to analyse associations between interpopulation distance matrices. The first principal component (PC1) from a PC analysis exhibited high and positive loadings for all traits in both sexes.

Individual walking-stick insects collected from both hosts ($n = 643$) were placed in the bottom of 500 ml plastic cups with one 12-cm host cutting from each host-plant species. These assays were initiated in the evening, and in the morning we recorded the position from which individuals were visible (for individuals choosing *Ceanothus* only, as the thin leaves of *Adenostoma* precluded measurement of this variable).

No-choice mating experiment

One male and one female were placed in a 10-cm Petri dish. At the end of one hour we scored whether the male and female were separate or copulating. We used walking-stick insects from eight populations, yielding 28 pairwise comparisons (13 same-host comparisons, 15 different-host comparisons). Sixteen sets of 16 individuals (one member of each sex from each population) were subjected to mating trials ($n = 64$ trials per pairwise comparison). Each individual was tested only once with an opposite-sex member from each of the eight populations, alternating the test order.

Statistical analysis of mating data

Copulation frequencies were analysed using logistic regression in a model that examined the dependence of copulation on male host, female host and an interaction term, with sexual isolation indicated by a significant interaction and significance assessed using a likelihood ratio test (LR). To test whether colour pattern contributed to sexual isolation, we used logistic regression to assess whether morph pair type (male and female the same or different colour-pattern morphs) in between-population crosses influenced the probability of copulation (using LR tests). To determine whether the degree of sexual isolation observed was dependent on which specific pair of populations was tested, we added male population and female population as terms in the previous logistic regression model and assessed the significance of the four-way interaction between all factors. We also tested for sexual isolation between populations for each pairwise comparison separately.

We calculated an index that summarizes the effects of sexual isolation (I_{PSI})¹², and used 5,000 re-samplings to calculate the standard deviation and significance of this index.

DNA sequencing

Individuals were collected from each of the populations used in the mating experiment (mtDNA (COI), $n = 40$, 5 individuals per population; nuclear DNA (ITS-2), $n = 23$, 2–4 individuals per population). For mtDNA, double-stranded PCR amplifications were performed with the primers S2183–A3014, S2195–A3014, S2183–A2887 and S2195–A2887 (ref. 13), which amplify most of the 3' half of the cytochrome oxidase I gene. For nuclear DNA, PCR amplifications were performed with the primers AED5.8F, 5'-TGTAAGTGCAGGACACATGAAC-3' and 28SBLD, 5'-TTCTTTTCTCC(C/G)CTTA(C/T)T(A/G)ATATGCTTAA-3'. Sequences are deposited in GenBank under accession numbers AF439805 to AF439820 (mtDNA) and AF459648 to AF459653 (nuclear DNA).

Phylogenetic analyses

Maximum likelihood trees were estimated using the HKY + G model of DNA substitution for mtDNA (COI) analyses and the F81 + G model for nuclear DNA (ITS-2) analyses, as these models gave the optimal fit to the data¹⁴. *Timema cristinae* from a different mountain (Ojala) was used as an outgroup for mtDNA analyses, whereas a closely related species (*T. monikensis*) was used as an outgroup for nuclear DNA analyses.

For mtDNA, analyses performed on unique haplotypes in PAUP 4.0b4b¹⁵ revealed that a molecular clock-constrained maximum likelihood tree (-ln likelihood = 915.07) did not differ significantly from a molecular clock-unconstrained tree with the same topology (-ln likelihood = 908.44; $\chi^2 = 13.26$, 14 d.f., $P > 0.50$)¹⁶. For nuclear DNA, a molecular clock was rejected (clock-unconstrained tree, -ln likelihood = 688.21; clock-constrained tree, -ln likelihood = 698.51; $\chi^2 = 0.60$, 6 d.f., $P < 0.05$).

The maximum likelihood mtDNA tree, showing bootstrap values from 200 replicates, was: (outgroup, (((P, L, H-HV-P): 82, L): 80, (P-VPC-VPA-OUTA-PR, (((H, H), (PR, PR-VPA-OUTA, VPC, VPC): 88): 64, (HV, H): 66): 90), (L, P): 94). The maximum likelihood nuclear DNA tree, showing bootstrap values from 200 replicates, was: (outgroup, P, (HV-L, (P-HV-L, L, (PR-VPC-OUTA-VPA-H, VPC): 54): 95): 73). Hyphens connect populations sharing a given haplotype, and colons precede bootstrap values. Populations using *Adenostoma* as a host are italicized. Eight allopatric populations were used.

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Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation

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Neuronal growth cones are guided to their targets by attractive and repulsive guidance cues¹. In mammals, netrin-1 is a bifunctional cue, attracting some axons and repelling others^{2–5}. Deleted in colorectal cancer (Dcc) is a receptor for netrin-1 that mediates its chemoattractive effect on commissural axons^{6,7}, but the signalling mechanisms that transduce this effect are poorly understood. Here we show that Dcc activates mitogen-activated protein kinase (MAPK) signalling, by means of extracellular signal-regulated kinase (ERK)-1 and -2, on netrin-1 binding in both transfected cells and commissural neurons. This activation is associated with recruitment of ERK-1/2 to a Dcc receptor complex. Inhibition of ERK-1/2 antagonizes netrin-dependent axon outgrowth and orientation. Thus, activation of MAPK signalling through Dcc contributes to netrin signalling in axon growth and guidance.

Signalling mechanisms triggered by netrin binding to Dcc are poorly understood. Dcc interacts with receptors of the Robo and UNC5H families, and adenosine A2b (refs 5, 8, 9), which are believed to modulate Dcc function. Beyond these cell-surface

interactions, one aspect of Dcc signalling already characterized is its role as a dependence receptor, inducing apoptosis in the absence of netrin-1 through a mechanism involving caspase cleavage of its cytoplasmic domain¹⁰. However, signalling events downstream of

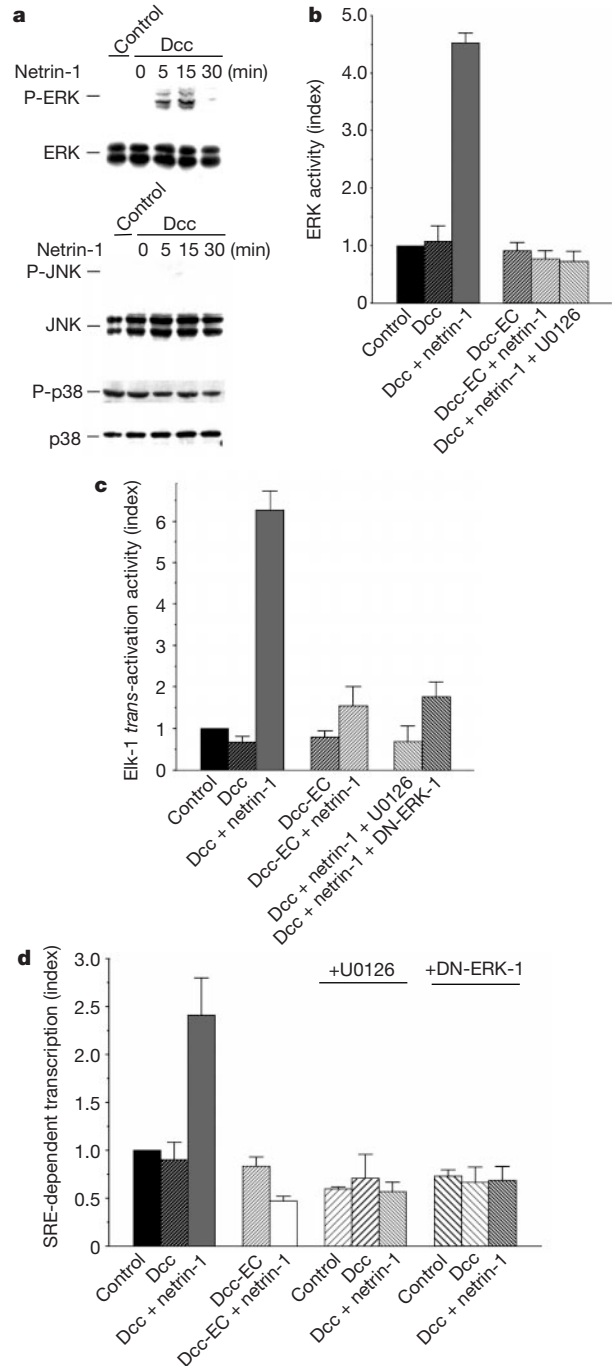


Figure 1 Dcc induces ERK-1/2 activation on netrin-1 binding. **a**, Western analysis of phosphorylated and total ERK-1/2, JNK and p38 in Dcc-transfected HEK 293 cells in response to 100 ng ml⁻¹ netrin-1. **b**, ERK kinase activity after 15 min of 100 ng ml⁻¹ netrin-1 in HEK 293 cells transiently expressing full-length or truncated Dcc (Dcc-EC; expression of the Dcc ectodomain). Control, 4 μM U0126. **c**, **d**, Elk-1 activation (**c**) or SRE-dependent gene expression (**d**) assessed by luciferase activity or SEAP expression, respectively, in HEK 293 cells transfected with full-length or truncated Dcc, with or without netrin-1 construct. Controls in **c** and **d**: co-transfection of dominant negative (DN) ERK-1, or addition of 4 μM U0126.